METHODS IN PLANT HISTOLOGY

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PLANT HISTOLOGY

BY

CHARLES J. CHAMBERLAIN, A.M., PH.D.

Instructor in Botany in the University of Chicago

SECOND EDITION

CHICAGO

The University of Chicago Press

1905

581.8
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PREFACE TO FIRST EDITION

This book has grown out of a course in histological technique conducted by the author at the University of Chicago. The course has also been taken by non-resident students through the Extension Division of the University. The *Methods* were published over a year ago as a series of articles in the *Journal of Applied Microscopy*, and have called out numerous letters of commendation, criticism, suggestion, and inquiry. The work has been thoroughly revised and enlarged by about one-half. It is hoped that the criticism and suggestion, and also the experience gained by contact with both resident and non-resident students, have made the directions so definite that they may be followed, not only by those who work in a class under the supervision of an instructor, but also by those who must work in their own homes without any such assistance.

More space has been devoted to the paraffin method than to any other, because it has been proved to be better adapted to the needs of the botanist. The celloidin method, the glycerine method, and free-hand sectioning are also described, and their advantages and disadvantages are pointed out.

The first part of the book deals with the principles of fixing and staining, and the various other processes of microtechnique, while in the later chapters these principles are applied to specific cases. This occasions some repetition, but the mere presentation of general principles will not enable the beginner to make good mounts.

The illustrations and notes in the later chapters are not intended to afford a study of general morphology, but they

merely indicate to students with a limited knowledge of plant structures the principal features which the preparations should show. The photomicrographs were made from the author's preparations by Dr. W. H. Knap, and Figs. 52, 57, and 59 (Figs. 61, 66, and 68 of second edition) were drawn by Miss Eleanor Tarrant; all other figures of plant structures were made from the author's drawings.

Corrections and suggestions will be heartily appreciated.

CHARLES J. CHAMBERLAIN.

CHICAGO,
June 1, 1901.

PREFACE TO THE SECOND EDITION

It is gratifying to the author to learn that the kindly reception accorded to *Methods in Plant Histology* has exhausted the edition. Since the first edition appeared, a little more than four years ago, laboratory methods have been greatly improved, and systematic experiments have made it possible to give much more definite directions for making preparations.

In the present edition much more attention has been given to collecting material. Professor Klebs's methods for securing various reproductive phases in the Algæ and Fungi have been outlined in a practical way. Methods for growing other laboratory material are more complete than in the earlier edition.

The paraffin method has been much improved, and the glycerine method has been almost entirely replaced by the Venetian turpentine method, to which a whole chapter is devoted. Other new chapters deal with microchemical tests, free-hand sections, special methods, and the use of the microscope.

The author is deeply indebted to his colleague, Dr. W. J. G. Land, for numerous suggestions and improvements in methods.

Corrections and suggestions will be heartily appreciated.

CHARLES J. CHAMBERLAIN.

CHICAGO,
July 1, 1905.

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PART I

CHAPTER I

APPARATUS

Some histological work, like temporary mounts, glycerine mounts, and free-hand sections, can be done with no expensive apparatus except a microscope. For most histological work, however, the following list includes only a necessary equipment: a microscope magnifying at least 400 diameters; a hand microtome; a microtome; a razor; a hone and a good razor strop; a paraffin bath and lamp; a turntable; a scalpel; a pair of needles; a pair of scissors; a pair of forceps; Stender dishes; bottles; minots or watch-glasses; a graduate (50 or 100 c.c.); pipettes; slides, 1×3 inches; round covers, 18 mm. or $\frac{3}{4}$ inch in diameter; and square covers, $\frac{7}{8}$ inch. Long covers, at least 22×50 mm., will be needed for some of the serial sections.

A convenient and effective microscope should have a rack and pinion coarse adjustment, a fine adjustment, two eyepieces (about one-inch and two-inch preferred), a low-power objective of two-thirds of an inch or a one-inch focus, a high-power objective of one-fifth or one-sixth of an inch focus, a double nosepiece, an iris diaphragm, and an Abbé condenser. A cheap and practical form is shown in Fig. 1, and similar instruments are for sale by all the leading companies.

Since the chemicals used in histological technique are likely to damage the stage and substage of the microscope, it is well to place upon the stage a piece of glass about three inches square. It is not necessary to fasten it to the stage, since it is merely for protection while examining slides which are wet with reagents. An old lantern slide or lantern slide cover is just right for this purpose. In our own laboratory we use for examining wet slides a cheap microscope with only a single low-power objective and a single ocular. Some manufacturers now furnish, free of charge,

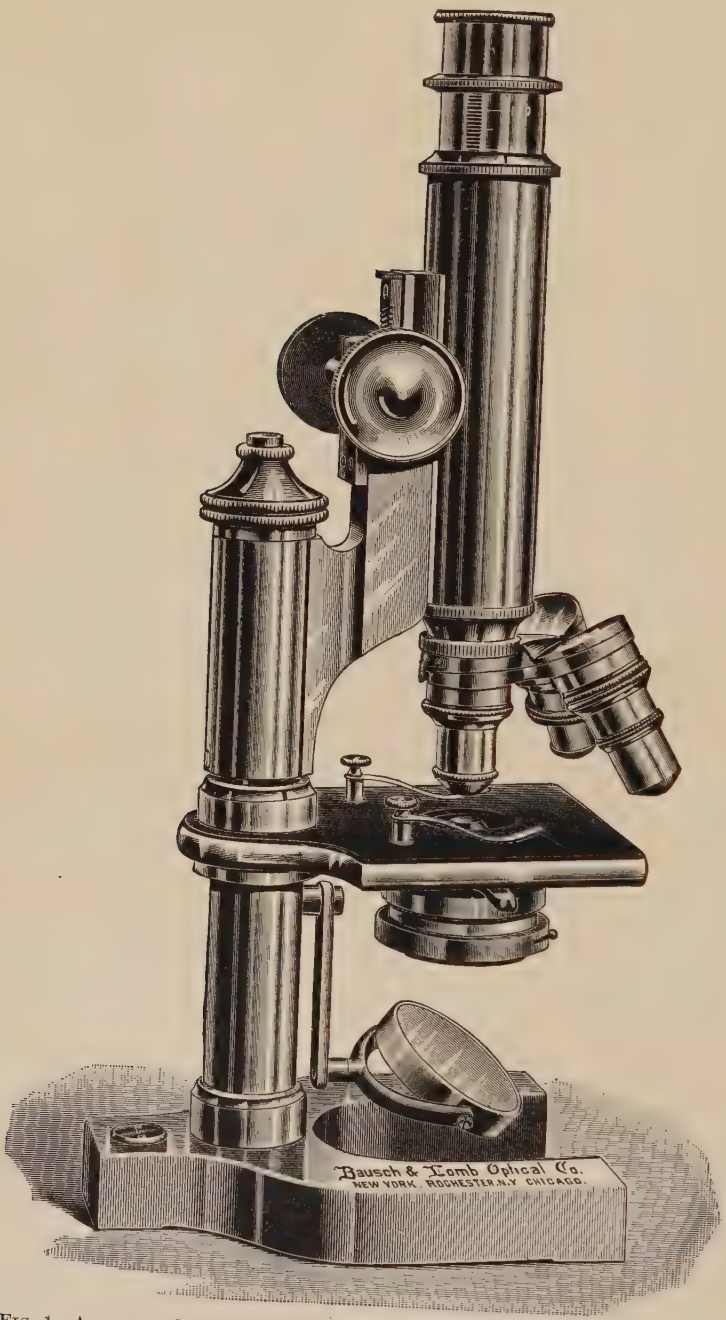

FIG. 1.—A compound microscope, with rack and pinion coarse adjustment, micrometer screw fine adjustment, triple nosepiece, iris diaphragm, and condenser.

a booklet, explaining the construction of the microscope and giving practical directions for its care and use.

The most convenient microtomes are rather expensive. While any sliding microtome, if kept in good order, is sufficient for the work described in this book, a rotary microtome will save an immense amount of time, if much paraffin sectioning is to be done. If there is to be only one microtome, it should be a sliding microtome, because celloidin sectioning and much other work cannot be done with a rotary microtome. A hand microtome (Fig. 2) would enable one to do the work described in the chapter on free-hand sections, but would be too uncertain for celloidin sections. The student's microtome

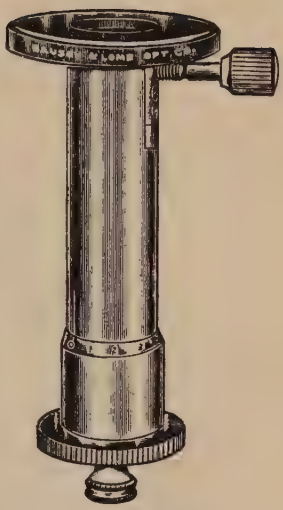

FIG. 2.—A convenient form of hand microtome.

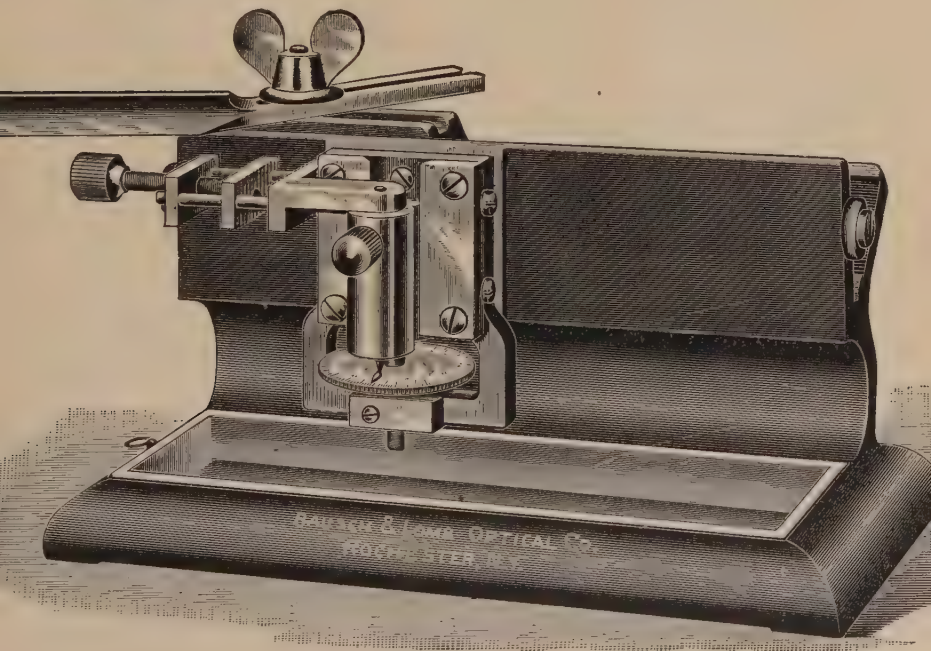

FIG. 3.—The student's microtome.

(Fig. 3) is quite inexpensive and does good work. It should be provided with a clamp (Fig. 4) which will hold any kind of a knife. Where expense is not too great an objection, a larger icrotome is preferable. The latest patterns of the Minot rotary microtome are almost ideal in their convenience and rapidity. We use this microtome almost exclusively for our paraffin work. Just as good sections, however, can be cut with a sliding microtome, and with very large or hard objects the greater stability of a sliding microtome of the Jung Thoma pattern is a distinct advantage. For large or hard objects the weakness of the student's microtome is evident from the figure.

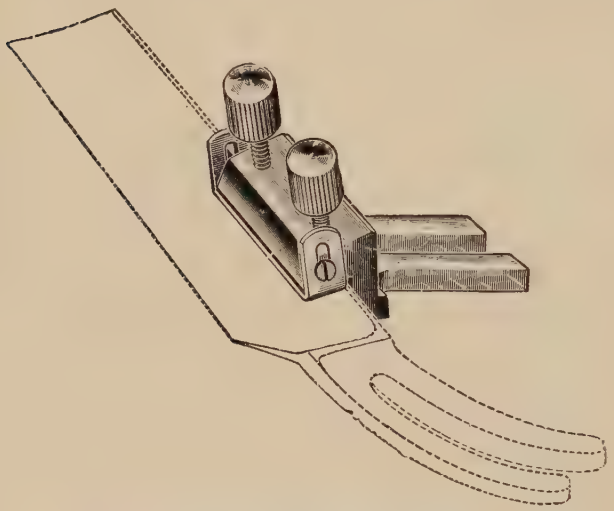

FIG. 4.—Clamp to hold the ordinary razor in the student's microtome.

The stout razors our grandfathers used to shave with are excellent for free-hand sectioning, for hand-microtome work, and even for cutting paraffin sections on the sliding microtome. The blade should be flat on one side (Fig. 5, *A*). Modern razors (Fig. 5, *B*) with delicate blades, though good to shave with, are worthless for cutting sections of plants. The razor is a necessity; if a microtome knife is wanted in addition, it should have a bevel about like that shown in Fig. 5, *A*. A short blade, two or three inches in length, is to be preferred to the longer ones, which are much more troublesome to sharpen.

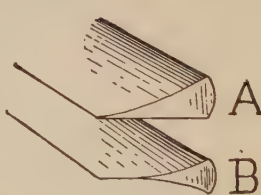

FIG. 5.

If manufacturers would make a clamp which would hold the

blade of the Gillette Safety Razor, its hard, even edge would doubtless be satisfactory for microtome sections, and the blades are so cheap that it would not be necessary to sharpen them when they became dull.

A good hone is a necessity. A yellow Belgian hone $10 \times 2\frac{1}{4}$ inches can be recommended. A rather soft hone for preliminary sharpening and a hard hone for finishing will save much time

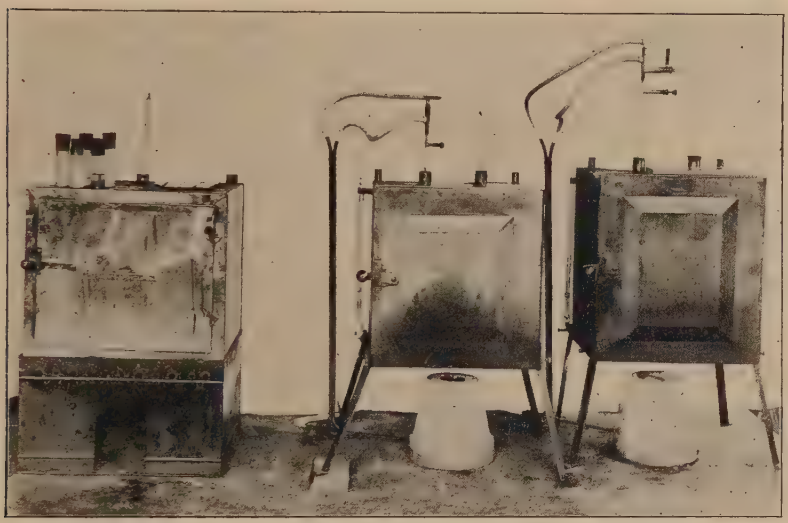

FIG. 6.—Three paraffin baths.

and patience. A very fine carborundum hone is good for the preliminary sharpening. If the second hone be quite hard and the finishing skilfully done, it may be unnecessary to use a strop. The best strops used by barbers are satisfactory for microtome knives. It is more convenient to have the strop mounted upon a board to keep it flat and rigid.

There are numerous forms of the paraffin bath. Those with a water-jacket, a thermometer, and a thermostat to maintain an even temperature are the most convenient where gas is available. An excellent equipment is shown in Fig. 6. The three baths are kept at different temperatures, the one on the left at 40° C., the

one in the middle at 48° C., and the other at 54° C. These baths are kept at a constant temperature, night and day, month after month. The destruction of the rubber, and consequent dangerous outflow of gas which would follow the "striking back" of the Bunsen burner, are guarded against by the metal tubes, to which the rubber tubes are attached. The top of the table is covered by a sheet of asbestos about one-fourth of an inch thick, and over this is a sheet of zinc. Baths set up in this way should be perfectly safe. In several years' experience with nearly a dozen baths running night and day we have never had a mishap. It is easier to keep the temperature constant in the larger baths. A bath which, if carefully watched, gives the very best results, can be made by any tinner, and is very inexpensive. The accompanying figures show the form and dimensions (Fig. 7).

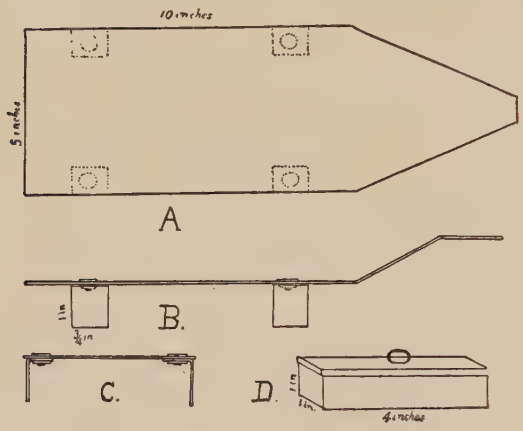

FIG. 7.—*A*, top view; *B*, side view; *C*, end view; *D*, box to contain the paraffin.

It is made of copper one thirty-second of an inch thick, but thicker copper is as good or better. There should be two boxes to contain the paraffin; the covers to the boxes should fit loosely. Any kind of a lamp may be used.

Since the principal use of the scalpel is to cut out paraffin blocks, trim them for the microtome, and handle the paraffin ribbons, the blade should be narrow and thin. The small blade of a pocket knife, if ground down to about one-half the usual thickness, is a good substitute.

Needles are used so constantly that it is well to have clamping holders. However, if it were not for the trouble of inserting and pulling out needles, nothing is better than a rather large handle whittled out from a piece of light pine.

Scissors are seldom used in the laboratory except for cutting out labels. In the field they are useful in preparing many kinds of material for the fixing agent. Small, rather stout scissors, with blades about $1\frac{1}{4}$ inches in length, are best for general purposes.

If there is to be but one pair of forceps, these should be strong enough to handle a slide without any danger of dropping it. For handling covers, a delicate pair is convenient.

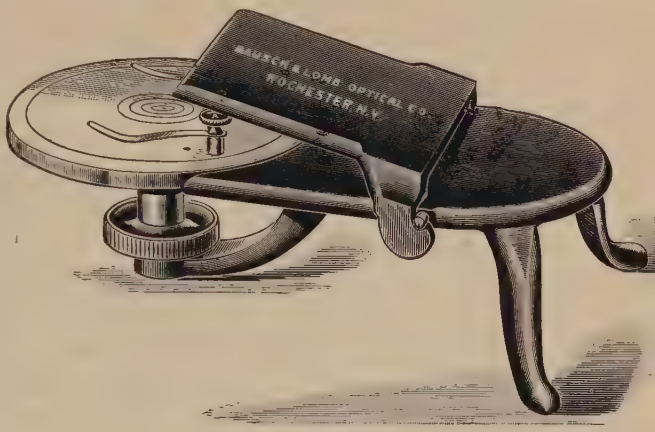

FIG. 8.—Turntable.

A serviceable form of turntable for glycerine mounts is shown in Fig. 8.

The more expensive turntables with devices for automatic centering present no practical advantages and the centering devices are frequently in the way.

Stender dishes are now very generally used for staining on the slide. The form shown in Fig. 9, *A*, is made just large enough to hold two slides, placed back to back, and hence requires only a minimum of the reagent. The cap in this form does not fit closely enough to keep absolute alcohol and xylol, but does very well for the other alcohols and stains. We do not believe that the convex cover is as good as a flat one. The form shown in Fig. 9, *B*, is the best for absolute alcohol and xylol, but even with this it is better to put a little vaseline or glycerine on the

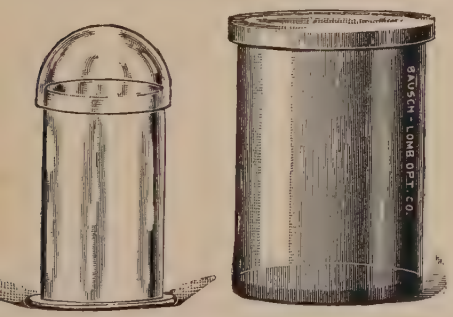

FIG. 9.—*A*, Naples jar; *B*, Stender dish.

cover to prevent any evaporation. Many staining-jars are upon the market, some of them affording more or less economy in reagents. Where a large number of slides must be stained at one time, a series of battery jars of diminishing sizes, placed one within the other like a nest of beakers, far surpasses any device now offered by the optical companies. The jars can be held in place with paraffin, or the inner jar may be partly filled with lead or mercury and so hold the others in place. Such a series, with the outer jar $4\frac{1}{2}$ inches in diameter and the inner one 2 inches in diameter, will accommodate forty slides without putting them back to back. Only 300 c.c. of stain is needed to fill such a series. Wide-mouthed bottles, though not convenient, give just as good results.

Solid watch-glasses, or Minots, as they are often called, are always useful. A student should have a dozen of these, if much attention is to be paid to the Venetian turpentine method or the glycerine method.

Slides and covers are a constant expense. Many slides now upon the market are imperfect. Beware of slides which are not perfectly flat. Of course, there should be no bubbles. "White" slides are to be preferred to those which appear greenish in the box. Very thin slides are to be avoided. Covers should be of medium thickness, but very thin covers are preferable to those which are of more than medium thickness. For mounts which are to be used with an immersion lens we prefer to have the cover of exactly the same width as the slide. The advantage is evident, since there is no danger of getting balsam on the cover when wiping off the immersion fluid; besides, one can put sections to the very edge of the slide and still be sure that they will be covered. Since most mounts for research work are mounted under long covers and are intended for examination with immersion lenses, we should recommend covers 25×50 mm., or even 25×60 mm., instead of 22×50 mm., the usual size. By consulting a catalogue, which will be furnished by any dealer, the beginner can determine what he needs to buy, and what he can find substitutes for, if it is necessary to be very economical.

CHAPTER II

REAGENTS

The number of reagents used in a fully equipped histological laboratory is constantly increasing. The following account includes those which are used constantly, and also a few which are used occasionally. *The Microtomist's Vade-Mecum*, by Lee, contains very complete formulæ for stains and other reagents. A list of reagents, with the quantities used by the average student in a three months' course in methods, is given in Chapter XXVI. "Stains and Staining" are described in the next chapter.

KILLING AND FIXING AGENTS

Usually the same reagent is used for both killing and fixing. The purpose of a killing agent is to bring the life-processes to a sudden termination, while a fixing agent is used to fix the cells and their contents in as nearly the living condition as possible. The fixing consists in so hardening the material that the various elements may retain their natural condition during all the processes which are to follow. Zoölogists often use chloroform or ether for killing an organism, and then use various fixing agents for various tissues. No promptings of humanity restrain the botanist from the vivisection of plants, but separate reagents for killing and fixing are sometimes used, e. g., material may be killed by placing it for a short time in Flemming's fluid, which is a very rapid killing agent, after which the fixing may be completed in a chromo-acetic solution, without any osmic acid, thus securing the advantages of a very rapid killing without the blackening which results from a prolonged treatment with a solution containing osmic acid.

Probably no process in microtechnique is in more urgent need of improvement than this first step of killing and fixing. Nearly all of our formulæ are merely empirical, for very few botanists

are expert chemists, and those who have the requisite knowledge of chemistry are interested in physiological problems rather than in microtechnique. The principal ingredients of the usual killing and fixing agents are: alcohol, chloroform, chromic acid, acetic acid, osmic acid, formic acid, picric acid, sulphuric acid, platinum chloride, iridium chloride, corrosive sublimate and formalin. We shall consider first

THE ALCOHOLS

a) **Ninety-five per cent. Alcohol.**—This is in quite general use for material which is needed only for rough work. It is extremely convenient, since it kills, fixes and preserves at the same time and needs no changing or washing. It really has nothing to recommend it for fine work. It causes protoplasm to shrink, but cell walls usually retain their position, so that 95 per cent. alcohol will do for free-hand sections of wood and many herbaceous stems; but even free-hand sections of tender stems, like young geraniums and begonias will look better if better reagents are employed. Alcohols weaker than 95 per cent. are not to be recommended as fixing agents, although 70 per cent. alcohol, or even 50 per cent., will preserve material for habit work. The time required for fixing in 95 per cent. alcohol is about the same as for absolute alcohol. The subsequent treatment is the same, except that material to be imbedded in paraffin or celloidin must be dehydrated in absolute alcohol. Material preserved in weaker alcohols and intended only for habit study may be kept in the reagent until needed for use. Unless some glycerine be added, material left in 95 per cent. alcohol becomes very brittle. Stems, roots, and similar objects may be kept indefinitely in a mixture of equal parts of 95 per cent. alcohol and glycerine.

Methyl alcohol, or wood alcohol as it is commonly called, serves equally well.

b) **Absolute (100 per cent.) Alcohol.**—This is a fair killing and fixing agent, but is rather expensive. It causes but little shrinking of the protoplasm, and is a time-saver if material is to be imbedded in paraffin. The time required for fixing in alcohol is very short. For small fungi, like *Eurotium*, 1 minute is long

enough. Root-tips of the onion, anther of the lily, and similar objects require 15–30 minutes. Larger objects may require an hour. No washing is necessary; consequently, material which is to be imbedded in paraffin is ready for clearing as soon as it is fixed; material like *Eurotium*, which is to be mounted in glycerine is brought directly into the stain.

Acetic acid is used with alcohols to counteract the tendency to shrink. One of the most successful of the alcohol combinations is

c) **Carnoy's Fluid.**—

Absolute alcohol	6 parts
Chloroform	3 parts
Glacial acetic acid	1 part

The penetration of the reagent is very rapid. An object like an onion root-tip is doubtless killed in less than a minute, and 10–15 minutes is long enough for fixing an object of this size. Wash in absolute alcohol, changing frequently, until there is little or no odor of acetic acid. For a root-tip, the entire process of fixing and washing should not require more than an hour. It is better to imbed in paraffin at once, but when this is not convenient the material may be transferred to 85 per cent. alcohol and then to 70 per cent., where it may be left until needed. Cyanin and erythrosin, fuchsin and iodine green, and similar combinations, give particularly brilliant staining after this reagent.

d) **Acetic Alcohol.**—Farmer and Shove recommend for fixing root-tips of *Tradescantia virginica* a mixture of two parts absolute alcohol and one part of glacial acetic acid. The mixture is allowed to act for 15–20 minutes, after which the acid is washed out with absolute alcohol and the material is imbedded as soon as possible.

THE CHROMIC-ACID GROUP

Chromic acid, or solutions with chromic acid as a foundation, are the most generally useful killing and fixing agents yet known to the botanist. A 1 per cent. solution of chromic acid in water gives good results, but it is better to use the chromic in connection with other ingredients, such as acetic acid, formic acid, osmic acid, etc. Chromic acid does not penetrate well, and this is one

reason why it is seldom used alone. Unfortunately it precipitates some liquid albuminoids in the form of filaments and networks, which may be mistaken for structural elements. In botanical work, acetic acid is nearly always mixed with chromic acid. The pickles of the dinner table show that acetic acid is a good preservative, and that it causes little or no shrinking. It penetrates rapidly, and is likely to cause swelling rather than shrinking, thus counteracting the tendency of chromic acid to cause plasmolysis. Acetic acid, not stronger than 1 or 2 per cent., in water is worth a trial. It may be washed out with water or alcohol.

It will be found convenient to have in the laboratory the following stock solution of chromo-acetic acid from which various solutions can be made as they are needed:

Chromic acid crystals	10 g.
Glacial acetic acid	10 c.c.
Water	1000 c.c.

To make a solution containing 0.5 g. of chromic acid and 2 c.c. of glacial acetic acid to 100 c.c. of water, add 50 c.c. of water to 50 c.c. of the stock solution, and then add to the weakened solution 1.5 c.c. of glacial acetic acid. Any desired proportions can be secured in a similar way. Weighing the crystals for every new proportion is more tedious. The proportions of the various ingredients, for the present at least, must be determined by experiment. With favorable objects like fern prothallia, *Spirogyra*, and other things which can be watched while the fixing is taking place, suitable proportions are rather easily determined, because specimens, after being placed in the reagent, may be examined at frequent intervals, and combinations which cause plasmolysis may be rejected and different proportions tried until satisfactory results are secured. For example, fern prothallia might be placed in the following solution: chromic acid, 2 g.; acetic acid, 1 c.c.; and water, 97 c.c. If plasmolysis takes place, weaken the chromic or strengthen the acetic, since the chromic has a tendency to produce contraction, and the acetic to cause swelling. A good fixing agent for fern prothallia can be made by adding 50 c.c. of water and 1 c.c. of glacial acetic acid to 50 c.c. of the stock

solution. This solution will cause practically no plasmolysis, and the fixing is thorough. A combination may be quite satisfactory for fern prothallia and still fail to give good results with *Spirogyra*, and a combination which succeeds very well with *Spirogyra* may not succeed at all with *Vaucheria*. For very critical work the most favorable proportions must be determined for the particular plant under investigation. In observing the effect of the fixing one can determine whether there is any noticeable plasmolysis or distortion, but whether the fixing is thorough can be determined only by noting how the tissues endure the subsequent processes. When the effect of the reagent cannot be observed directly, it is well to make a free-hand section and thus determine whether plasmolysis takes place. It is not safe to judge the action of a fixing agent by the appearance of sections cut from material which has been imbedded in paraffin, because shrinking of the cell contents often takes place during the transfer from absolute alcohol to the clearing agent or during infiltration with paraffin, and sometimes even during later processes. When in doubt as to proportions, we should suggest 2 c.c. chromic acid, 3 c.c. acetic acid, and 300 c.c. water as a good formula for most purposes.

The time required for fixing undoubtedly varies with different objects, but even a delicate object, like *Spirogyra*, which is penetrated immediately, should remain in the fixing fluid for 6–10 hours. Most botanists leave material like onion root-tips and lily ovaries in the chromo-acetic acid for 12 to 24 hours. Some recommend longer periods. Christman, in his work on rusts, left material for three days in Flemming's fluid, a much more vigorous agent than the chromo-acetic acid. We have often imbedded material which had been in chromo-acetic acid for several days, and it seemed to have suffered no injury. It is well known that zoölogists allow fixing agents like Müller's fluid and Erlicki's fluid to act for weeks before the material is passed on to the next stage, and it may well be questioned whether botanists have not made a mistake in allowing the chromic solutions to act for so short a time. More rapid penetration, and consequently more immediate killing, can be secured if the reagent is

kept warm (30° to 40° C.). The warming also shortens the time required for fixing, but heating before the protoplasm has been killed is likely to exaggerate kinoplasmic activity. Of course, heating after the protoplasm is dead could not produce such a result.

After fixing is complete, all reagents containing chromic acid as an ingredient should be washed out with water. Running water is desirable, and where this is not convenient the water must be changed frequently.

One hour should be long enough for filamentous algæ and fungi, which are immediately penetrated by the water. For fern prothallia, 2 or 3 hours in running water, or in water changed frequently, is sufficient. Onion root-tips or lily anthers should be washed in 3 or 4 hours; lily ovaries at the fertilization period should not require more than 6 hours. Any material should be sufficiently washed in 6–24 hours, and this time may be shortened about one-half by using lukewarm water. The water should be changed as soon as it becomes in the least discolored by the chromic acid. When the material can remain in the water for half an hour without causing any discoloration, the washing out process is sufficiently complete. If the washing has not been thorough, the hæmatoxylins will not stain, but the anilins will stain in spite of incomplete washing.

Some of the formulæ are as follows:

a) **Stock Chromo-Acetic Solution.**—

Chromic acid	1 g.
Glacial acetic acid	1 c.c.
Water	100 c.c.

This solution has been used quite extensively in embryological work upon the higher plants. It fixes thoroughly, but often causes plasmolysis in cells with large vacuoles.

b) **Weak Chromo-Acetic Solution** (Schaffner's formula).—

Chromic acid	0.3 g.
Acetic acid	0.7 g.
Water	99 c.c.

This has also been used in embryological work. It causes little or no plasmolysis. Difficult work, like *Aster* heads and ripe

Capsella pods, cuts more readily after this reagent than after the stronger solution.

c) **Medium Chromo-Acetic Solution.**—

Chromic acid	0.5 g.
Glacial acetic acid	0.1 g.
Water	100 c.c.

This is a useful formula. The chromic is too strong for some algæ, but for fern prothallia and most liverworts the solution is quite successful.

d) **Flemming's Fluid** (weaker solution).—

A.	1 per cent. chromic acid	25 c.c.
	1 per cent. acetic acid	10 c.c.
	Water	55 c.c.
B.	1 per cent. osmic acid	10 c.c.

Keep the mixture A made up, and add B as the reagent is needed for use, since it does not keep well. This fluid is quite expensive on account of the osmic acid. For cytological work it gives as good results as any fixing agent which has yet been thoroughly tested. It is especially recommended for chromosomes, centrosomes, achromatic structures, and mitotic phenomena in general. Material should be in very small pieces one-eighth of an inch square, or in thin slices one-eighth of an inch or less in thickness, for the fluid penetrates poorly. The blackening due to the osmic acid may be removed by peroxide of hydrogen just before the slide is passed from the alcohol into the stain. Harper and Holden, in their work on *Coleosporium*, recommended 4 hours on the slide in a 3 per cent. solution of the peroxide of hydrogen. According to Miss Merriman, the linin in the nuclei of onion root-tips is not so well preserved in this solution, but the arrangement of the chromatin granules is brought out with greater distinctness .Flemming's safranin, gentian-violet, orange combination gives its most brilliant results after this reagent. A small quantity of osmic acid added to any of the chromic acid mixtures will facilitate the staining of kinoplasmic structures with gentian-violet, but as fixing agents it seems possible that the

traditional superiority of mixtures containing osmic acid has been overestimated.

e) **Merkel's Fluid.**—

Equal volumes of a 1.4 per cent. solution of chromic acid and a 1.4 per cent. solution of platinic chloride.

This is also an expensive reagent. It is recommended for mitotic phenomena, but does not seem to equal Flemming's solution.

f) **Hermann's Fluid.**—

1 per cent. platinic chloride . .	15 parts
Glacial acetic acid	1 part
2 per cent. osmic acid . . .	4 or 2 parts

This is the most expensive fixing agent yet discovered, and for botanical purposes it does not seem to be any better than the cheaper chromic mixtures. It is mentioned here with chromic mixtures because it originated as a variation of Flemming's fluid, the platinic chloride being substituted for the chromic acid.

PICRIC ACID

Use a saturated solution in water or 70 per cent. alcohol. One gram of picric acid crystals will saturate about 75 c.c. of water or alcohol. This reagent penetrates well and does not make the material brittle. It is to be recommended when difficulty is anticipated in the cutting. If used cold, the time varies from 1 to 24 hours, depending upon the character of the tissue and size of the specimen. If used hot (85° C.), 5 or 10 minutes will be sufficient. Material should be washed in 70 or 50 per cent. alcohol. Water is injurious, and some even go so far as to avoid aqueous stains, unless the material has been thoroughly washed. The washing should be continued until the material appears whitish and the alcohol no longer becomes tinged with yellow. Picro-carmine gives its best result after this reagent. Picric acid can be combined with various other fixing agents, and so we have picro-sulphuric acid, picro-nitric acid, picro-chromic acid, picro-chromic-sulphuric acid, picro-osmic acid, picro-alcohol, and picro-corrosive sublimate. The picric acid in all mixtures should be rather strong.

CORROSIVE SUBLIMATE

Corrosive sublimate, or bichloride of mercury, is soluble in water and in alcohol. About 5 g. will make a saturated solution in 100 c.c. of water. It is somewhat more soluble in alcohol, but for practical purposes 5 g. in 100 c.c. of 50 per cent. alcohol may be regarded as a saturated solution. Corrosive sublimate used alone does not give as good results as when mixed with acetic acid, chloroform, or picric acid. Fixing is very rapid, the material being fixed almost as soon as it is penetrated by the fluid. Material which is at all transparent, like some ovules and the endosperm of gymnosperms before the formation of starch, becomes opaque as soon as fixed, and so the time needed for fixing is easily determined. From 10 minutes to one hour should be sufficient for onion root-tips or lily ovaries. Smaller or larger objects require shorter or longer periods. When used hot (85° C.) the fixing is much more rapid. Filamentous algæ or fungi are simply dipped into the fixing agent and immediately taken out. One minute is enough for onion root-tips, and two minutes is enough for a lily ovary at the fertilization period.

Wash out aqueous solutions with water and alcoholic solutions with alcohol. Material fixed in aqueous solutions may be washed in several changes of water and then passed successively through 15, 35, 50 and 70 per cent. alcohol; material fixed in alcoholic solutions should be washed in 50 per cent. alcohol, and then should be transferred to 70 per cent. alcohol. At this point one should make it sure that the reagent has been washed out. Add to the 70 per cent. alcohol a little of the iodine solution used in testing for starch. It will impart a brownish color to the alcohol, but the color will disappear in a few seconds, and the alcohol will become clear if any corrosive sublimate remains. Add more and more iodine until the brown color fails to disappear. The washing is then complete. If the washing is incomplete, the preparations will be disfigured by crystals of corrosive sublimate. Camphor may be used instead of iodine to hasten the washing, but it does not give a color reaction. Material

should be imbedded as soon as possible, since it gets brittle if allowed to remain in the alcohol.

Kinoplasmic structures do not stain well with genetian-violet, but safranin and the hæmatoxylins stain almost as well as after chromic acid mixtures, and the carmines give their most brilliant stains, due to the formation of mercuric carminate.

The following formulæ are merely suggestive:

a) **Corrosive sublimate and acetic acid.**—

Corrosive sublimate	3 g.
Glacial acetic acid	3 c.c.
Alcohol (or water)	100 c.c.

b) **Corrosive sublimate, acetic acid and picric acid.**—

Corrosive sublimate	5 g.
Glacial acetic acid	5 c.c.
Picric acid, saturated solution in 50 per cent. alcohol	100 c.c.

c) **Corrosive sublimate and picric acid** (Jeffrey's solution).—

Corrosive sublimate, saturated solution in 30 per cent. alcohol	3 parts
Picric acid, saturated solution in 30 per cent. alcohol	1 part

It would be worth while to try other combinations.

d) **Formalin** is an excellent preservative, often preserving the blue and red colors as well as the structure of objects. A 2 or 4 per cent. solution in water is good for filamentous algæ. A 4 per cent. solution in water is good for habit material of gymnosperms and many similar things. The material may simply be put into the reagent and left until needed for use. For class use formalin material should be washed in water for several minutes, because the fumes are irritating to the eyes and mucous membranes. After a thorough washing in water any of the usual stains may be used.

GENERAL HINTS ON FIXING

It is very desirable that the fixing agent penetrate quickly to all parts of the object. For this reason material should be in small pieces. The best fixing agents do their best work near the

surface of the piece. Small objects like *Azolla* and fern prothallia may be thrown into the fixing agent entire; even larger objects, which, like the anthers of *Lilium*, are easily penetrated, may also be put in without any cutting. Most objects larger than quarter of an inch cubes should be trimmed with a sharp knife or razor; some knowledge of the structures concerned is essential before one can trim material with unvarying success.

Some objects, although small, cause trouble in various ways. Many buds are hairy and will not sink; if such things are dipped quickly in strong alcohol, they will usually sink. If rather large air bubbles prevent the material from sinking, as in case of perichætical leaves of some mosses and involucral leaves of liverworts, a little dissection or a careful snip with the scissors will obviate the difficulty. If an air-pump is available, the bubbles are easily removed. Heating followed by rapid cooling is recommended by Pfeiffer and Wellheim for removing air.

It is often asked whether fixing agents really preserve the actual structure of cell contents. It must be admitted that some things, notably the liquid albuminoids, are much modified in appearance, but the most competent observers are now inclined to believe that such delicate objects as chromosomes, centrosomes, the achromatic figure, and even the structure of protoplasm, can be studied with confidence from material which has been fixed, imbedded, and stained. Recent study of these objects in the living condition has strengthened this confidence.

It is certain that we have not yet found the ideal fixing agent for cell contents. Such an agent must not be solvent of any of the cell contents, must penetrate rapidly, must preserve structures perfectly, and must harden so thoroughly that every detail shall remain unchanged during the subsequent processes of dehydrating, clearing, imbedding, sectioning, and staining.

DEHYDRATING AGENTS

Objects which are to be imbedded in paraffin or celloidin, and also all other objects which are to be mounted in balsam, must be dehydrated, i. e., they must be freed from water. The slightest

trace of water is ruinous. Alcohol is used almost exclusively for dehydrating. The process must be gradual. If material has been fixed in an aqueous solution, it must pass through a series of alcohols of increasing strength, beginning with about 15 per cent. alcohol. If the fixing agents contain 50 per cent. alcohol, it is not necessary to use any alcohol weaker than 50 per cent. in washing or dehydrating. The following series is sufficiently complete for the most critical work: 15, 35, 50, 70, 85, 95, and 100 per cent. alcohol. For material like onion root-tips, an hour or two in each grade is sufficient. Objects as large as 1 c.c. cubes should remain in each grade 12–24 hours. These periods are merely suggestive. Some large objects are easily penetrated, fixed, and dehydrated, while some small objects are refractory. After material has been in 15 per cent. alcohol for the proper period, the 15 per cent. alcohol is poured off and the 35 per cent. poured on, and the other grades are used in the same way. The alcohols, except the 100 per cent., can be used repeatedly. The absolute should be changed once or twice to insure perfect dehydration. The absolute alcohol should not be used again for this purpose, but it should be saved and used for rinsing slides after the paraffin ribbons have been dissolved off with xylol or turpentine. Be sure that the bottle or Stender dish is *absolutely dry* before putting absolute alcohol or any clearing agent into it, and then keep the bottle tightly corked or the Stender dish closely covered. The importance of excluding moisture cannot be exaggerated. Grades of alcohol below 95 per cent. alcohol are made up from the 95 per cent. alcohol.

Formulæ for Alcohols.—The following formulæ will enable anyone to make the other grades of alcohol from 95 per cent. alcohol and water.

95	15	95	35	95	50	95	70	95	85
	80		60		45		25		10

The above are the formulæ for 15 per cent., 35 per cent., 50 per cent., 70 per cent., and 85 per cent. alcohol. Any other grade can be got in the same way. In the first formula subtract 15

from 95; the result, 80, is the number of cubic centimeters of water which must be added to 15 c.c. of 95 per cent. alcohol in order to obtain 15 per cent. alcohol. The mixture contains 95 c.c. of 15 per cent. alcohol. If more or less than 95 c.c. of the mixture is needed, take proportional parts of 15 and 80. This simple method is a time-saver, but if the bottles or Stender dishes are to be filled frequently, it will be a still further saving of time to use a long label (Fig. 10) and, after pouring in the 95 per cent. alcohol, draw a line showing how high it reaches, and then, after pouring in the water, draw another line. The next time it is necessary to fill the bottles merely pour in 95 per cent. alcohol until it reaches the first line, and then pour in water until it reaches the second line. It is not necessary to use distilled water, if pure drinking-water is available.

FIG. 10

Synthol is used like alcohol, and many believe it to be a good substitute.

Some very minute objects, like bacteria and the smaller Cyanophyceae, may be dehydrated by heating them until all water is drawn off.

CLEARING AGENTS

Clearing agents are so named because they render objects transparent. When clearing agents are used to precede infiltration with paraffin, the clearing is merely incidental, the real purpose being to replace the dehydrating agent with a solvent of paraffin. The clearing is useful, even in this case, because it indicates when the replacing has become complete.

When the clearing agent is used to precede infiltration with paraffin, the material should always be most thoroughly dehydrated with absolute alcohol before beginning with the clearing agent. When the clearing agent is used to clear sections or small objects just before mounting in balsam, absolutely perfect dehydration is not necessary with all clearing agents. Bergamot oil, carbolic acid, and Eycleshymer's clearing fluid (equal parts of bergamot oil, carbolic acid, and cedar oil) will clear readily

from 95 per cent. alcohol. Sections to be cleared in xylol or clove oil should be dehydrated in absolute alcohol.

Xylol.—In our opinion, xylol is the best clearing agent to precede infiltration with paraffin. After the material has been dehydrated, it should be brought gradually into xylol. Use three mixtures of absolute alcohol and xylol before putting the material into pure xylol. These mixtures may be used repeatedly. The proportions are given below. The periods are for ovaries of lily at the fertilization period.

Alcohol 3 parts, xylol 1 part . .	2 hours
Alcohol 2 parts, xylol 2 parts . . .	2 hours
Alcohol 1 part, xylol 3 parts . .	2 hours
Pure xylol	2 hours

The pure xylol must not be used again for this purpose, but should be saved and used for dissolving paraffin ribbons when staining on the slide.

Xylol is an excellent agent for clearing sections just before mounting in balsam. Preparations cleared in xylol dry quickly and this is a decided advantage. Xylol evaporates so rapidly that one must take care not to let the sections become dry before the balsam is applied. Gentian-violet is the only important stain which does not clear well in xylol. Thin sections perfectly dehydrated, should clear in a few seconds; a minute should be sufficient even for thick sections.

Chloroform.—Some botanists use chloroform to precede the infiltration with paraffin. In the later stages of infiltration it is more easily removed than xylol. It seems to possess no other advantages, and for clearing sections just before mounting in balsam it is inferior to xylol or clove oil. Its value in hardening celloidin and as a fixing agent entitles it to a place in the histological laboratory.

Cedar Oil.—It is not always easy to get good cedar oil. If the stuff offered for sale looks like turpentine and smells like it, it is worthless for histological purposes. Good cedar oil has a slightly amber tint, the color resembling a weak clove oil. It should have the pleasant odor of cedar wood. The very expensive cedar

oil used with immersion lenses is not needed for clearing or for preceding infiltration with paraffin. It is claimed that material cleared in cedar oil does not become so brittle as that cleared in xylol or chloroform.

Clove Oil.—This is an excellent agent for clearing sections and small objects just before mounting in balsam. It clears more readily than xylol. When the absolute alcohol has deteriorated so that xylol no longer clears the sections, clove oil may still clear with ease. While clove oil will clear from 95 per cent. alcohol, it is better to use absolute. Since preparations cleared in clove oil harden slowly, it is a good plan, to keep them at a temperature of 30° to 40° C. for about 24 hours. Some recommend a dip in xylol after clearing in clove oil to hasten the hardening. Gentian-violet is somewhat soluble in clove oil, and this fact makes it possible to secure a beautiful differentiation, because the stain is extracted from some elements more rapidly than from others. The stain may be extracted completely from the chromosomes during the metaphase and still remain bright in the achromatic structures. After the desired differentiation has been attained the clove oil should be replaced by a few drops of cedar oil or some other clearing agent which is not a solvent of gentian-violet, since the continued action of clove oil would cause the preparation to fade. Do not use a Stender dish for clove oil, but keep it in a 50 c.c. bottle. Put on a few drops, and immediately drain them off in such a way as to remove the alcohol as completely as possible. Then flood the slide and pour the clove oil back into the bottle, repeating the process until the proper differentiation has been reached. Replace the clove oil with cedar oil and mount in balsam. With stains not soluble in clove oil, the cedar oil is not necessary.

Clove oil is used in removing the celloidin matrix from celloidin sections. It is useless as an agent to precede infiltration with paraffin.

Eycleshymer's Clearing Fluid.—This is a mixture of equal parts of bergamot oil, cedar oil and carbolic acid. It clears readily from 95 per cent. alcohol and, consequently, is useful in

clearing celloidin sections when it is desirable to preserve the celloidin matrix. In sections stained with hæmatoxylin, or hæmatoxylin and eosin, the stain may be removed completely from the matrix by the use of acid alcohol, and the matrix can be preserved by clearing from 95 per cent. alcohol.

It is not intended that the mixture should be used to precede infiltration with paraffin.

Other Clearing Agents.—Bergamot oil, carbolic acid, turpentine, benzine, gasoline, and other reagents have been tried for clearing, but none seem to be worth more than a warning mention.

MISCELLANEOUS REAGENTS

Canada Balsam is used almost exclusively for mounting. Very thick balsam is very disagreeable to handle and makes unsatisfactory mounts. Very thin balsam, in drying out, allows bubbles to run under the cover. Xylol is cheaper than balsam and, consequently, the balsam on the market is likely to be too thin for immediate use. The stopper may be left out until the the balsam acquires the proper consistency. Material cleared in clove oil or cedar oil may be mounted directly in xylol balsam. It is not necessary that the clearing agent should be also the solvent of the balsam.

Paraffin should be of at least two grades, a soft paraffin melting at 40° to 45° C., and a hard paraffin melting at 53° to 54° C. Grübler's paraffin and most imported paraffins melt at the temperature indicated on the wrappers. The melting-point indicated on the wrappers of paraffins sold by some American dealers does not enable one to make even a guess as to the real melting-point. One prominent optical company sells a paraffin marked 70° C., which usually melts at 52° to 55° C. The fact that the price rises with the melting-point may explain the discrepancy.

Paraffin may be used repeatedly. Keeping it in the liquid condition in the bath month after month is an advantage, since it becomes more and more tenacious and homogeneous.

Glycerine, glycerine jelly, Venetian turpentine and gold size are described in the chapter on "The Glycerine Method."

Celloidin is described in the chapter on "The Celloidin Method." The reagents already described are noted further in connection with specific applications. Reagents used in making microchemical tests are described in the chapter on temporary mounts.

A list of reagents with suggestions in regard to quantities and prices will be found in Chapter XXVI.

CHAPTER III

STAINS AND STAINING

During the past five years the science and art of staining have not improved so rapidly as might have been expected. Nearly all cytological papers state that preparations were stained in the Flemming triple stain or in Haidenhain's iron-hæmatoxylin. That so little progress has been made is probably due to the fact that these two combinations have become so well known, and the method of using them so thoroughly developed, that excellent results are secured with scarcely any preliminary experimentation. Other stains which, properly developed, might have given better results in particular cases, have been neglected. There is abundant room for experimentation in the use of mordants and in the effect of the same stain or combination after various fixing agents.

Stains may be classified in various ways; e. g., there are three great groups of stains—the Carmines, the Hæmatoxylins, and the Anilins. Stains may be classified as basic and acid, or they may be regarded as general and specific. A general stain affects all the elements, while a specific stain affects only certain elements or stains some elements more deeply than others. Stains which show a vigorous affinity for the nucleus have been called nuclear stains, and those which affect the cytoplasm more than the nucleus have been termed plasma stains. Of course, such stains are specific.

We shall consider some of the more important hæmatoxylins, carmines, and anilins, reserving general directions and theoretical questions for another chapter. The formulæ are largely empirical. Some of those given here are taken from *The Microtomist's Vade-Mecum* (Lee), which is easily the most complete compendium of stains and other reagents concerned in microtechnique. It is to be regretted that botanists have no book of this character, but it must be confessed that we have not the material for such an

extensive work. Other formulæ are from *Botanical Microtechnique* (Zimmermann) and from Stirling's *Histology*, and still others are from our own laboratory. The directions for using a stain apply to stains made up according to the formulæ which are given here, and may need modification if other formulæ are employed. It is hoped, however, that the directions will give the student sufficient insight into the *rationale* of staining to enable him to make any necessary modifications.

THE HÆMATOXYLINS

The most important hæmatoxylins are Delafield's hæmatoxylin, Erlich's hæmatoxylin, Boehmer's hæmatoxylin, Mayer's hæmalum, Haidenhain's iron alum-hæmatoxylin, and Kleinenberg's hæmatoxylin.

All the hæmatoxylins mentioned above contain alum, and, according to Mayer, who has written the most important work on hæmatoxylin stains,[1] "The active agent in them is a compound of hæmatin with alumina. This salt is precipitated in the tissues, chiefly in the nuclei, by organic and inorganic salts there present (e. g., by the phosphates) and perhaps also by other organic bodies belonging to the tissues." These salts are fixed in the tissues by the killing and fixing agent, and when the stain is applied a chemical combination results. Hæmatoxylins stain well after any of the fixing agents described in the preceding paper, but they are most effective when used after members of the chromic-acid series.

Delafield's Hæmatoxylin.—"To 100 c.c. of a saturated solution of ammonia alum add, drop by drop, a solution of 1 g. of hæmatoxylin dissolved in 6 c.c. of absolute alcohol. Expose to air and light for one week. Filter. Add 25 c.c. of glycerine and 25 c.c. of methyl alcohol. Allow to stand until the color is sufficiently dark. Filter, and keep in a tightly stoppered bottle." (Stirling and Lee.) The addition of the glycerine and methyl alcohol will precipitate some of the ammonia alum in the form of small

[1] "Ueber das Färben mit Hämatoxylin," *Mittheilungen aus der Zoologischen Station zu Neapel*, **10**: 170–186, 1891, and "Ueber Hämatoxylin, Carmin und verwandten Materien," *Zeitschrift für wissenshaftliche Mikroscopie*, **16**: 196–220, 1899.

crystals. The last filtering should take place 4 or 5 hours after the addition of the glycerine and methyl alcohol.

The solution should stand for at least two months before it is ready for using. This "ripening" is brought about by the oxidation of hæmatoxylin into hæmatin, a reaction which may be secured in a few minutes by a judicious application of peroxide of hydrogen.

Transfer to the stain from any alcohol or from water. The length of time required is exceedingly variable. Sometimes sections will stain deeply in 3 minutes, but it is often necessary to stain for thirty minutes or even longer. This stain may be diluted with several times its own volume of water; when this is done, the time required is correspondingly long, but the staining is frequently more precise. The length of time required will be fairly uniform for all material taken from the same bottle. This fact indicates that the washing process, which follows killing and fixing, is an important factor; if the washing has been thorough, the material will stain readily; but if the washing has been insufficient, the material may stain slowly or not at all. The washing is particularly important when the fixing agent contains an acid. Transfer from the stain to water. Distilled water is neither necessary or desirable. Some writers recommend washing for 24 hours, but this seems unnecessary; an hour is usually enough, and a few minutes is often sufficient. Precipitates are often formed when slides are transferred directly to alcohol from this stain, and sometimes even after washing in water. A few gentle dips in acid alcohol (5 drops of HCl to 100 c.c. of 70 per cent. alcohol) will usually remove the precipitates. This extracts the stain more rapidly from other parts than from the nuclei, and hence gives a good nuclear stain, while at the same time it removes any disfiguring precipitates. Some prefer to stain for a very short time and use no acid alcohol, but, as a rule, it seems best to overstain and then differentiate in this way, because sharper contrasts are obtained. Transfer from acid alcohol to 70 per cent. alcohol and leave here until a rich purple color replaces the red due to the acid. Since small quantities of

the acid alcohol are carried over into the 70 per cent. alcohol, it is well to add a *drop* of ammonia now and then to neutralize the effect of the acid. Too much ammonia is to be avoided, for it gives a disagreeable bluish color with poor differentiation, probably on account of the precipitation of alumina. The preparation is now dehydrated in 95 per cent. and then in absolute alcohol, cleared in xylol or clove oil, and mounted in balsam.

If a double stain is preferred, after the acid has been washed out, stain for about 1 minute in eosin, erythrosin, orange G, or any stain affording a good contrast, and then transfer directly to 95 per cent. alcohol, dehydrate in absolute alcohol, and proceed as usual. The method of staining in safranin and Delafield's hæmatoxylin is given in the chapter on "Free-Hand Sections."

The following is a general schedule for staining paraffin sections on the slide in Delafield's hæmatoxylin:

1. Stain (from water or any alcohol) . . . . . 5 minutes
2. Rinse in water . . . . . 5 minutes
3. Acid alcohol . . . . . 1 second
4. 70 per cent. alcohol . . . . . 1 minute
5. 95 per cent. and 100 per cent. alcohol . . . 30 seconds each
6. Xylol . . . . . 1 minute
7. Mount in balsam.

If, after rinsing in water, the stain is evidently too weak, put the slide or section back into the stain until it appears overstained. Give the slide a single dip into the acid alcohol, transfer it quickly to the 70 per cent. alcohol, and then examine it; if it still appears overstained, give it another dip in the acid alcohol, and repeat the process until the stain is what you want. After the hæmatoxylin is just right, apply a contrast stain, if you wish to double-stain. It is a good plan to move the slide gently to and fro in the absolute alcohol. Before transferring to the xylol, wipe the alcohol from the back of the slide, or at least rest the corner of the slide upon blotting-paper for two or three seconds, in order that you may not carry over so much alcohol into the xylol, and thus impair this rather expensive reagent. The

slide may also be moved gently to and fro in the xylol. Add a drop of balsam and a cover. Since the xylol is very volatile, this last step must be taken quickly. If blackish spots appear, they are usually caused by the drying of sections before the balsam and cover are added; if there are whitish spots or an emulsion-like appearance, the clearing is not thorough; this may be caused by poor xylol (or other clearing agent); by absolute alcohol which is considerably weaker than its name implies (the absolute alcohol must test at least as high as 99 per cent., and ought to test as high as 99.5 per cent., if xylol is to be used for clearing); or the difficulty may be caused by passing too quickly through the absolute alcohol and xylol, or may even be caused by moisture on the cover-glass.

Delafield's hæmatoxylin is the most generally useful stain in the hæmatoxylin group. It brings out cellulose walls very sharply, and consequently is a good stain for embryos and the fundamental tissue system in general. With safranin it forms a good combination for the vascular system, the safranin giving the lignified elements a bright red color, while the hæmatoxylin stains the cellulose a rich purple. It is a good stain for chromatin, and the achromatic structures show up fairly well, but can be brought out much better by special methods. Archesporial cells and sporogenous tissue are very well defined if proper care be taken. Lignified and suberized walls and also starch and chromatophores stain lightly or not at all. Whenever you are in doubt as to the selection of a stain for general purposes, we should advise the use of Delafield's hæmatoxylin.

Haidenhain's Iron Alum-Hæmatoxylin.—This stain was introduced by Haidenhain in 1892 and has gained a well-deserved popularity with those engaged in cytological work. Two solutions are used, and they are never mixed:

A. 1½ to 4 per cent. aqueous solution of ammonia sulphate of iron.
B. ½ per cent. aqueous solution of hæmatoxylin.

The first solution acts as a mordant, i. e., it does not stain, but prepares the tissue for the action of the second solution.

Transfer to the iron-alum from water; allow this solution to

act for 2 hours; wash in water 5 minutes, and then stain in the ½ per cent. hæmatoxylin 10 hours or over night. Rinse in water 5 minutes and treat for a second time with the iron-alum, which now rapidly extracts the stain. The action of the iron-alum should be watched under a microscope, and when the chromosomes of karyokinetic figures appear sharply defined, the slide should at once be placed in water and washed for at least an hour, since a very little of the iron-alum, if left in the tissue, will cause the preparation to fade. If staining for details other than nuclei, the slide must be transferred to water as soon as the desired effect is produced. After the washing in water, dehydrate in 95 per cent. and in absolute alcohol, clear in clove oil or xylol, and mount in balsam. This stain is excellent for the filamentous algæ and fungi, and it keeps well in all the mounting media. Erythrosin, fuchsin, and orange G are good contrast stains. Apply the second stain after the last washing in water. The second stain should be rather light.

The times given above must not be accepted as final. Many prefer to wash in water for several hours after the first immersion in iron-alum. Some think that 4 hours is enough for the entire process. Many put the slide into iron-alum in the morning and finish the process in the afternoon. A plan which has proved convenient and very successful is to put the slide into the iron-alum in the morning, let it wash in water during the afternoon, leave it in the ½ per cent. of hæmatoxylin over night, and finish the preparation the next morning. It is a long process, requiring care, patience, and judgment, but it is worth the effort.

Chromosomes, centrosomes, and pyrenoids take a brilliant black, or, if the second treatment with iron-alum be more prolonged, a blue black or purple. Achromatic structures stain purple, but the stain can be extracted while it is still bright in the chromosomes. Lignified, suberized, and cutinized structures stain lightly or not at all. Cellulose does not stain so deeply as with Delafield's hæmatoxylin. Archesporial cells and early stages in sporogenous tissue stain gray. Many details which are not so brilliantly colored often show good definition.

Mayer's Hæm-Alum.—Hæmatoxylin, 1 g., dissolved with gentle heat in 50 c.c. of 95 per cent. alcohol and added to a solution of 50 g. of alum in a liter of distilled water. Allow the mixture to cool and settle; filter; add a crystal of thymol to preserve from mold. (Lee.)

It is ready for use as soon as made up. Unless attacked by mold it keeps indefinitely. Transfer to the stain from water. It is seldom necessary to stain for more than 10 minutes, and 4 or 5 minutes is generally long enough. As a rule, better results are secured by diluting the stain (about 1 c.c. to 10 c.c. of distilled water) and allowing it to act for 10 hours or over night.

This is a good stain for the nuclei of filamentous algæ and fungi, since it has little or no effect upon cell walls or plastids. Wash thoroughly in water and transfer to 10 per cent. glycerine. Specimens may be mounted in Venetian turpentine.

The transfer to Venetian turpentine is made as follows: Allow the 10 per cent. glycerine to concentrate just as when mounting in glycerine; wash the glycerine out in 95 per cent. alcohol; then transfer the material to 10 per cent. Venetian turpentine and allow the turpentine to concentrate in an exsiccator. The mounts are as firm as balsam mounts and sealing is unnecessary.

Erlich's Hæmatoxylin.—

Distilled water	50 c.c.
Absolute alcohol	50 c.c.
Glycerine	50 c.c.
Glacial acetic acid	5 c.c.
Hæmatoxylin	1 g.
Alum in excess.	

Keep it in a dark place until the color becomes a deep red. If well stoppered, it will keep indefinitely. Transfer to the stain from 50 per cent. or 35 per cent. alcohol. Stain 5–30 minutes. Since there is no danger from precipitates and the solution does not overstain, it is not necessary to treat with water or with acid alcohol, but the slide may be transferred from the stain to 70 per cent. alcohol. Eosin, erythrosin, or orange G are good contrast stains. Jeffrey uses safranin and Erlich's hæmatoxylin for woody tissues.

Boehmer's Hæmatoxylin.—

A	Hæmatoxylin	1 g.
	Absolute alcohol	12 c.c.
B	Alum	1 g.
	Distilled water	240 c.c.

The solution A must ripen for two months. When wanted for use, add about 10 drops of A to 10 c.c. of B. Stain 10–20 minutes. Wash in water and proceed as usual.

Cellulose walls take a deep violet. The closing membrane (torus) of the bordered pits of conifers will usually stain deeply in about 15 minutes. Lignified, suberized, and cutinized structures stain slightly or not at all. When they do stain, the color is not violet, but a light yellow or brown.

Kleinenberg's Hæmatoxylin.—This stain has had wide use, but it is now largely replaced by better formulæ. It is mentioned here merely because it is on the shelves of so many laboratories. It is doubful whether it is equal to Delafield's in any kind of botanical work. A good description is given in the *Quarterly Journal of the Microscopical Society*, **74**: 208. 1897.

THE CARMINES

This group of stains, immensely popular several years ago, has rapidly lost favor among botanists as newer stains and combinations have appeared. Botanists have not given the carmines a fair trial in recent years. It is possible that it would be worth while to try again, especially after fixing agents containing mercury. When it is desirable to stain in bulk, nothing has been found which will serve better than the carmines. Only three of these stains will be considered:

Greenacher's Borax Carmine.—

Carmine	3 g.
Borax	4 g.
Distilled water	100 c.c.

Dissolve the borax in water and add the carmine, which is quickly dissolved with the aid of gentle heat. Add 100 c.c. of 70 per cent. alcohol and filter. (Stirling.)

The following is a slightly different method for making this stain from the ingredients mentioned above: Dissolve the borax in water, add the carmine, and heat gently for 10 minutes; after the solution cools, add the alcohol and filter; let the solution stand for 2 or 3 weeks, then decant and filter again.

Stain the material in bulk from 50 per cent. alcohol 1 to 3 days, then treat with acid alcohol (50 c.c. of 70 per cent. alcohol + 2 drops of hydrochloric acid) until the color becomes a clear red; this may require only a few hours, but may take 2 or 3 days. The material may then be passed through the rest of the alcohols (6–24 hours each), cleared, imbedded and cut. After the sections are fastened to the slide, the paraffin should be dissolved off with xylol. The balsam and cover may be added immediately, or the xylol may be rinsed off with alcohol and a contrast stain may be added.

Alum Carmine.—A 4 per cent. aqueous solution of ammonia alum is boiled 20 minutes with 1 per cent. of powdered carmine. Filter after it cools. (Lee.)

Stain from water 12–24 hours and wash in water. No acid alcohol is needed, since the solution does not overstain.

Alum Cochineal.—

Powdered cochineal	50 g.
Alum	5 g.
Distilled water	500 c.c.

Dissolve the alum in water, add the cochineal, and boil; evaporate down to two-thirds of the original volume, and filter. Add a few drops of carbolic acid to prevent mold. (Stirling.)

Stain as with alum carmine. A few years ago it was a very common practice to stain in bulk in alum cochineal and counterstain on the slide with Bismarck brown.

THE ANILINS

Many of the most brilliant and beautiful stains yet discovered belong to this group. These stains are so numerous that we shall not attempt to mention even their names, but shall consider only those which are in most common use by botanists. The

following general formula has proved to be fairly satisfactory for most anilins, but the formulæ mentioned in describing the different stains are usually to be preferred.

General Formula.—Make a 10 per cent. solution of anilin oil in 95 per cent. alcohol; when the anilin oil is dissolved, add enough water to make the whole mixture about 20 per cent. alcohol; add 1 g. of cyanin, erythrosin, safranin, gentian-violet, etc., to each 100 c.c. of this solution.

The anilins keep well in balsam but not in glycerine. Xylol is a good clearing agent for all of them, but clove oil should be used with gentian-violet. Unfortunately, some of them do not give permanent stains. Some are acid, some basic, and some neutral.

The rapidity with which sections must be transferred from one fluid to another makes many of them more difficult to manage than the hæmatoxylins or the carmines, but the stains are so valuable that even the beginner should spend most of his time with the anilins.

Many anilins stain quite deeply in 1 to 20 minutes, but if the stain washes out during the dehydrating process, stain longer, even 10 to 24 hours if necessary. Often the brilliancy of the stain can be increased by leaving the slide for 5 minutes in a 1 per cent. solution of permanganate of potassium before staining. The permanganate acts as a mordant.

The following are the more important anilins now in use by botanists. The directions apply to solutions made up according to the formulæ given with the different stains.

Safranin.—Two safranins are sold by dealers, one soluble in water and the other soluble in alcohol. The alcoholic is somewhat soluble in water and the aqueous is somewhat soluble in alcohol, but both make better solutions when used with their intended solvents.

The best aqueous solution is simply a 1 per cent. solution in distilled water.

The alcoholic solution is made by dissolving 1 g. of the alcoholic safranin in 100 c.c. of 95 per cent. or absolute alcohol

and, after the safranin is completely dissolved, add 50 c.c. of distilled water.

According to Flemming, dissolve 0.5 g. of alcoholic safranin in 50 c.c. of absolute alcohol, and after 4 days add 10 c.c. of distilled water.

A method which we have used for three years with good results is to make a 1 per cent. solution of the aqueous safranin in distilled water; then make a 1 per cent. solution of the alcoholic safranin in 95 per cent. alcohol; then mix equal volumes of the two solutions. This makes a strong solution of safranin in about 50 per cent. alcohol.

An anilin safranin may be made according to the general formula.

Since the anilin and aqueous solutions seem to have no positive advantages, the directions given here apply to the alcoholic solutions and the mixture of the two solutions.

Transfer to the stain from any grade of alcohol or from water. Paraffin sections are most conveniently transferred to the stain from 95 per cent. alcohol after the xylol or turpentine used in dissolving the paraffin has been rinsed off in alcohol. Celloidin sections are transferred to the stain from 70 per cent. alcohol. Free-hand sections which have been fixed in 95 per cent. or absolute alcohol are passed directly into the stain. Free-hand sections fixed in alcoholic mixtures are transferred to the stain from the medium in which the washing has been completed. Free-hand sections fixed in any of the chromic acid fixing agents are transferred to the stain from water.

The time required for staining varies with the tissue, the fixing agent, and the quality of the stain. In general, it may be said that 2 hours is a minimum and 24 hours a maximum. If the staining be too prolonged, delicate structures, like starch grains, crystals, and various cell constituents, may wash out. The mere fact that the whole section does not wash off does not mean that everything is fastened to the slide. On the other hand, it is difficult to get a sharp differentiation. In staining a vascular bundle, one should be able to wash the safranin from the cellulose

walls and still leave a brilliant red in lignified structures. For paraffin sections, 3–6 hours will usually be sufficient. It is a good practice to put the slides into the stain in the morning and finish the mounts any time in the afternoon. For free-hand and celloidin sections of woody tissues longer periods are better.

From the stain transfer to 50 per cent. alcohol. If the sections are deeply stained, and sufficient differentiation is not secured within 5 or 10 minutes, a drop of hydrochloric acid added to 50 c.c. of the alcohol will hasten the extraction of the stain. If staining vascular tissue, draw the stain from the cellulose walls, but stop before the liquified walls begin to fade. If staining mitotic figures, draw the stain from the spindle, but stop before the chromosomes begin to weaken. When the desired differentiation has been reached, wash out the acid in 50 per cent. alcohol, if acid has been used. One or 2 minutes should be sufficient.

If the safranin is to be used alone, dehydrate in 95 per cent. and absolute alcohol, clear in xylol or clove oil, and mount in balsam. If a second stain is to be added, transfer from the 50 per cent. alcohol to any alcoholic stain. If the second stain is an aqueous stain, it is better to rinse the slide or sections for a minute in water before applying the stain.

Safranin is the most generally useful of all the red stains, and, fortunately, it is quite durable. Lignified, suberized, and cutinized membranes stain red, as do also the chromosomes, nucleoli, and centrosomes.

Acid Fuchsin.—Use a 1 per cent. solution in water or in 70 per cent. alcohol. The solution in alcohol is preferable if sections are to be mounted in balsam. This stain often acts with great rapidity, two or three minutes being sufficient. The method for using acid fuchsin with woody tissues is given in the chapter on "Free-Hand Sections." In staining embryo-sacs, pollen grains, and such structures, longer periods are better. Stain one or two hours, and then differentiate in a saturated solution of picric acid in 70 per cent. alcohol. This may require 30 seconds or even several minutes. Rinse in 70 per cent. alcohol until a bright red replaces the yellowish color due to the acid, and then proceed as usual.

Eosin.—This has long been a favorite stain, but for most purposes it has been replaced by similar stains giving better differentiation. The dry stain is made in two forms, one for aqueous and the other for alcoholic solution. Each should be used with its intended solvent. Make a 1 per cent. solution in alcohol or water.

For algæ or fungi to be mounted in glycerine use the aqueous solution and stain for several hours; treat with 1 per cent. acetic acid for several seconds, and then wash the acid out thoroughly in water. Place in 10 per cent. glycerine, and allow the glycerine to concentrate. According to Lee, the glycerine should be slightly alkaline. The alkalinity can be brought about by adding half a gram of common salt to 100 c.c. of the 10 per cent. glycerine. We have found that eosin keeps well when the glycerine is acidulated with about 1 c.c. glacial acetic acid to 100 c.c. of 10 per cent. glycerine.

For staining paraffin sections, the alcoholic solution is better. One or 2 minutes is usually sufficient, and it is not necessary to use acid.

Hæmatoxylin and eosin, and methyl blue and eosin, are good combinations. The eosin should follow the other stain.

Erythrosin.—This is really an eosin, but there is some difference in the method of manufacturing. It is a more precise and a more transparent stain than eosin and is to be preferred for nearly all staining of paraffin sections. Make a 1 per cent. solution in distilled water or in 70 per cent. alcohol. It gives good results when made up according to the general formula.

Erythrosin stains rapidly, 30 seconds to 3 minutes being sufficient. When used in combination with other stains, erythrosin should come last.

Magdala Red.—At least two Magdala reds are sold by dealers, one the *echt* (genuine) Magdala red, and the other simply Magdala red. The latter is much cheaper and, in our experience, much superior to the *echt* stain. The directions apply to the cheaper stain.

For staining algæ which are to be mounted in Venetian turpentine, use a 1 per cent. solution in 85 or 95 per cent. alcohol.

Dilute the stain about one-half with 95 per cent. alcohol and allow it to act for 6–8 hours. Transfer to 10 per cent. Venetian turpentine and allow the turpentine to concentrate in an exsiccator.

In staining sections to be mounted in balsam, the same stain may be used, but it is better to dilute it one-half with water. Stain for 6–24 hours, dehydrate in 95 per cent. and in absolute alcohol, clear in clove oil and mount in balsam.

Magdala red stains lignified, suberized, and cutinized structures, and also chromosomes, centrosomes, nucleoli, and pyrenoids. It is likely to overstain, but the differentiation is easily secured by placing the finished mounts upon a white background in the direct sunlight. When the desired differentiation has been reached, it is better to avoid direct sunlight, although the mounts do not seem to fade in the ordinary light of a room.

Except for special purposes, it is better to use this stain in combination with blue, green, or violet.

Gentian-Violet.—This is one of the most important stains in the botanical laboratory. It may be made according to the general formula for anilin stains, but that solution does not keep well. A 1 per cent. solution in distilled water keeps indefinitely and seems to be as good, if not better than the anilin solution.

Dip the slide in water before transferring to the stain. From 1–30 minutes will be sufficient. The brilliancy of the stain in achromatic structures may often be increased by leaving the slide for 2–5 minutes in a 1 per cent. solution of permanganate of potassium before applying the stain. The stain washes out very rapidly in alcohol. Transfer from the stain to 95 per cent. alcohol, which should be allowed to act only 2 or 3 seconds, and is used merely to save the more expensive absolute alcohol. If the slide be moved gently to and fro in the absolute alcohol, 4 or 5 seconds should be long enough to complete the dehydration. Holding the slide in one hand, pour on a few drops of clove oil, and immediately drain off in such a way as to carry off the alcohol. This clove oil should not be used again. Then flood the slide repeatedly with clove oil, pouring the clove oil back into the bottle. A 50 c.c. bottle of clove oil is large enough. About

100 mounts can be cleared with 50 c.c. of clove oil. The clove oil is a solvent of gentian-violet, but it dissolves the stain from some structures more rapidly than from others; e. g., the stain may be completely removed from the chromosomes while it is still bright in the achromatic structures. The clove oil should be drained off, and it is usually better to replace it with a drop or two of cedar oil, which is not a solvent of gentian-violet. Some dip the slide in xylol before mounting in balsam. If the clove oil is not removed, its action will continue and the preparation will fade.

Gentian-violet is the best stain yet discovered for achromatic structures. Chromatin, in many of its stages, is also stained. Starch is stained rather deeply and chromatophores rather lightly. Granular constituents of the cell are variously stained. This stain is of great importance in giving clear views of minute structures, but is of little value in indicating the nature of cell constituents.

Cyanin.—This stain is also called Quinoleïn Blue and Chinolin Blue. Dissolve 1 g. of cyanin in 100 c.c. of 95 per cent. alcohol and add 100 c.c. of water. The cyanin would not dissolve in 50 per cent. alcohol. We have not found Grübler's cyanin very satisfactory with the above formula. With the general formula the Grübler's cyanin will not dissolve. We use a cyanin prepared by H. A. Metz & Co., 122 Hudson street, New York. This cyanin dissolves completely when made up according to the general formula. It stains rapidly, 5–10 minutes usually being sufficient. Chromosomes take a deep blue, but the spindle is only slightly affected. Lignified structures stain blue, while cellulose walls are scarcely affected and the stain is easily washed out.

Iodine Green.—Use a 1 per cent. solution in 70 per cent. alcohol. Stain for an hour, rinse in 70 per cent. alcohol, dehydrate in 95 per cent. alcohol and absolute alcohol, clear in xylol or clove oil, and mount in balsam. If the stain washes out too rapidly and does not give sufficient differentiation, stain longer, over night or even 24 hours.

Lignified structures stain green, but, after proper washing, cellulose is scarcely affected. A bright green may be left in the chromosomes after all the stain has been washed out from the spindle.

Acid fuchsin, erythrosin, and eosin are good contrast stains for mitotic figures. Acid fuchsin or Delafield's hæmatoxylin are good for cellulose walls.

Methyl Green.—A 1 per cent. solution in water is good for staining lignified structures. Lee recommends that the solution be acidulated with acetic acid. This is not necessary for staining lignified membranes nor for staining chromosomes. Methyl green has long been a favorite stain for living tissues. It is more easily controlled than iodine green, especially in double staining to differentiate lignified and cellulose walls.

Acid Green.—Make a solution according to the general formula, or simply make a 1 per cent. solution in water. This stains cellulose walls and achromatic structures, but scarcely affects lignified walls or chromosomes.

Anilin Blue.—Strong alcoholic solutions are best for botanical work. Even though the dry stain may be intended for aqueous solution, make a 1 per cent. solution in 85 or 95 per cent. alcohol.

This stain is particularly valuable for algæ. Directions for using it with algæ are given in the chapter on "The Venetian Turpentine Method."

Anilin blue is also useful for staining mitotic figures. Directions are given on page 80.

Orange G.—Make a 1 per cent. solution in water.

This is a plasma stain. It is often used with safranin and gentian-violet. It is useful in staining sections of woody tissues which have been stained in safranin and Delafield's hæmatoxylin, if the cell contents have been preserved. It may also be used as a background in staining sections containing bacteria.

Bismarck Brown.—Use a 2 per cent. solution in 70 per cent. alcohol.

This is a good stain for cellulose walls, although it is not so

precise as hæmatoxylin. Embryo-sacs stained in one of the carmines are improved by 1 or 2 minutes' staining in Bismarck brown. Material fixed in alcohol stains better than that which has been fixed in reagents containing chromic acid. A faint background of Bismarck brown is quite effective in staining sections containing bacteria.

Nigrosin.—Make a 1 or 2 per cent. solution in water. A few drops of this solution to a watch-glass full of water stains filamentous algæ or fungi in 1–3 hours. It keeps well in glycerine or Venetian turpentine. It also keeps well in balsam, but it is of little value in staining microtome sections.

COMBINATION STAINS

Sometimes preparations are stained with a single stain, selected to emphasize some particular feature, but in the great majority of cases two or more stains are used. In staining a vascular bundle, one stain may be selected which stains the xylem, but not the phloëm, while another of a different color stains the phloëm, but not the xylem, thus affording a sharp contrast. In staining mitotic figures, one stain may stain the chromosomes, while another of a different color may be used to stain the spindle.

Success in double staining can be obtained only by noting the effect of each stain upon the various plant structures.

Flemming's Safranin, Gentian-Violet, Orange.—Safranin has long been a famous stain for karyokinesis. This triple combination was published in 1891, but its value in plant cytology was not thoroughly appreciated until five or six years later, when its application was developed to a high degree of perfection by various investigators of the Bonn (Germany) school.

According to Flemming, stain 2 or 3 days in safranin (dissolve 0.5 g. safranin in 50 c.c. absolute alcohol, and after 4 days add 10 c.c. distilled water); rinse quickly in water; stain 1–3 hours in a 2 per cent. aqueous solution of gentian-violet; wash quickly in water, and then stain 1–3 minutes in a 1 per cent. aqueous solution of orange G. Transfer from the stain to absolute alcohol, clear in clove oil, and mount in balsam.

The following formulæ and method seem to be better for mitotic phenomena in plants: Make a 1 per cent. solution of alcoholic safranin in absolute or 95 per cent. alcohol, and after the safranin is completely dissolved, add an equal volume of a 1 per cent. solution of aqueous safranin in water, thus making a 1 per cent. solution of safranin in 50 per cent. alcohol. Use a 1 per cent. aqueous solution of gentian-violet, and a 1 per cent. aqueous solution of orange G.

Transfer to the stain from any grade of alcohol or from water. Paraffin sections are naturally transferred to the stain from 95 per cent. alcohol after the turpentine or xylol used in dissolving away the paraffin has been rinsed off. Stain 2–24 hours. If the period be too short, the washing out is so rapid that it is difficult to stop the differentiation at the proper point, and besides, the red is likely to be less brilliant. Rinse in 50 per cent. alcohol until the stain is properly differentiated. Leave the slide in the 50 per cent. alcohol until the stain is washed out from the spindle and cytoplasm, but stop the washing out before the chromosomes begin to lose their bright red color. If the washing out takes place too slowly, treat with slightly acidulated alcohol (1 drop of HCl to 50 c.c. of 50 per cent. alcohol) for a few seconds. The acid must be removed by washing for 15–30 seconds in alcohol which has not been acidulated.

Then dip the slide 3 or 4 times into water and stain in gentian-violet. The time required is so variable that definite instructions are impossible. The gentian-violet should stain the spindle, but not the chromosomes. If the stain be too prolonged, it may be impossible to get it out from the chromosomes and still leave it bright in the spindle. If the period be too short, the stain will wash out from the spindle. For mitotic figures in the germinating spores of the liverwort, *Pellia*, 30 minutes is not too long. In this case, the stain washes out easily from the chromosomes without the use of acid, and the spindle takes a rich violet which is not easily washed out. In embryo-sacs of *Lilium*, try 10 minutes. In pollen mother-cells try 5–10 minutes. For root-tips try 2–10 minutes. Chromatin in the early prophasis and in

telophases will stain with the violet, and the violet will not wash out, but in phases in which fully formed chromosomes are visible the violet can be washed out if the period has not been too long. In extreme cases, acid alcohol may be used to remove the violet.

Remove the slide from the gentian-violet and dip it 3 or 4 times in water and then stain 30 seconds to 1 minute in orange G. The orange stains cytoplasm and at the same time washes out gentian-violet.

Transfer from the orange G to 95 per cent. alcohol, dipping the slide a few times in this merely to save the absolute alcohol. Dehydrate in absolute alcohol 3–30 seconds.

Clear in clove oil, as already described in the paragraph on gentian-violet. The achromatic structures are very likely to fade unless the clove oil is followed by cedar oil or xylol.

Safranin and gentian-violet are often used without the orange. In this case, transfer from the gentian-violet directly to 95 per cent. alcohol, and proceed as before.

Cyanin and Erythrosin.—Make both solutions according to the general formula for anilins, but note what was said about cyanin in the paragraph on page 43.

Stain in cyanin 5–10 minutes or longer; rinse quickly in 50 per cent. alcohol, and then stain 30 seconds to 1 minute in erythrosin. If the cyanin washes out, stain for 1 hour, and if it still washes out, omit the rinsing in alcohol and transfer directly from the cyanin to the erythrosin.

The erythrosin may be used first; in this case stain for 5 minutes in erythrosin, transfer directly to cyanin, and stain for about 10 seconds. Dehydrate in 95 per cent. and in absolute alcohol, clear in xylol or in clove oil, and mount in balsam.

Chromosomes and nucleoli stain blue and achromatic structures red. Lignified structures stain blue and cellulose walls red The various cell constituents are often sharply differentiated. It was this combination which suggested the now obsolete terms, "cyanophilous" and "erythrophilous."

Magdala Red and Anilin Blue.—Make both solutions as directed in the chapter on "The Venetian Turpentine Method."

Stain 2 hours or more in Magdala red, dip in 95 per cent. alcohol to rinse off the stain, and then stain 2–10 minutes in the anilin blue. Dip in 95 per cent. alcohol to rinse off the stain, and treat for a few seconds with alcohol slightly acidulated with hydrochloric acid (one drop to 50 c.c. of 95 per cent. alcohol). In the acid alcohol the blue will become more intense, but the red would soon be extracted. Wash in 95 per cent. alcohol to remove the acid, dehydrate in absolute alcohol, clear in clove oil, and mount in balsam. It is better that the preparation should be overstained with the red. As in staining algæ, place the finished mount on a white ground in direct sunlight to reduce the red in case of overstaining, but keep the mount away from direct sunlight after the desired differentiation has been attained.

It may be worth while to try very weak solutions, using only 8–10 drops of the strong stain to 100 c.c. of 95 per cent. alcohol.

Acid Fuchsin and Iodine Green Mixtures.—Two solutions are kept separate, since they do not retain their efficiency long after they are mixed:

A	Fuchsin acid	0.1 g.
	Distilled water	50 c.c.
B	Iodine green	0.1 g.
	Distilled water	50 c.c.
C	Absolute alcohol	100 c.c.
	Glacial acetic acid	1 c.c.
	Iodine	0.1 g.

Mix equal parts of A and B. Transfer to the stain from water. The proper time must be determined by experiment. Twenty-four hours might be recommended for a trial. Transfer from the stain directly to solution C and from C to xylol.

Another formula:

A.	Acid fuchsin	0.5 g.
B.	Iodine green	0.5 g.

Mix a pipette full of A with a pipette full of B; stain 2 to 8 minutes; transfer to 85 per cent. or 95 per cent. alcohol, dehydrate rapidly, clear in xylol, and mount in balsam. Both these formulæ are good for karyokinesis.

Acid Fuchsin and Methyl Green.—Both may be used in 1 per cent. aqueous solutions.

For mitotic figures, stain in green for about an hour, wash in water or alcohol until the green is extracted from the spindle, and then stain for about one minute in the fuchsin. Dehydrate in 95 and 100 per cent. alcohol, clear in xylol or clove oil, and mount in balsam. If the green washes out, stain longer; if it is not readily extracted from the spindle, shorten the period. If the fuchsin stains the chromosomes, shorten the period, and lengthen it if the fuchsin washes out from the spindle. The chromosomes should take a brilliant green and the spindle a bright red.

Delafield's Hæmatoxylin and Erythrosin.—Stain first in the hæmatoxylin, and after that stain is satisfactory, stain for 30 seconds or 1 minute in erythrosin. This is a good combination, and, for most plant structures, gives a far better differentiation than the traditional hæmatoxylin and eosin, since the erythrosin has all the advantages of the eosin and is more transparent. Orange G is also a good stain to use with Delafield's hæmatoxylin.

Directions for staining in safranin and Delafield's hæmatoxylin are given in the chapter on "Free-Hand Sections."

Haidenhain's Iron-Hæmatoxylin and Orange G.—This hæmatoxylin is very satisfactory when used alone. A light staining in orange G, however, sometimes improves the mount. After the last washing in water, stain for about 30 seconds in orange G. Eosin, erythrosin and nearly all plasma stains fail to increase the effect of a good stain in iron-hæmatoxylin.

CHAPTER IV

GENERAL REMARKS ON STAINING

Many things may be examined alive without killing, fixing, staining, or any of those processes. A filament of *Spirogyra* shows the chromatophore nicely if merely mounted in a drop of water; the nucleus may be visible and the pyrenoids can usually be located. Of course, such a study is necessary if one is to understand anything about the plant, and in an elementary class this might be sufficient, but a drop of iodine solution applied to the edge of the cover would emphasize certain details, e. g., the starch would appear blue, the nucleus a light brown, and the cytoplasm a lighter brown. This illustrates at least one advantage to be gained by staining; it enables us to see structures which would otherwise be invisible, or almost invisible.

SELECTION OF A STAIN

With so many stains at our disposal, it at once becomes a problem just which stain or combination to use in each particular case. Beautiful and instructive preparations occasionally result from some happy chance, but uniform success demands skill and judgment in manipulation, and also a knowledge of the structures which are to be differentiated. Let us take a vascular bundle for illustration. Safranin stains the xylem a bright red, but, with judicious washing, is entirely removed from the cambium and cellulose elements of the phloëm. A careful staining with Delafield's hæmatoxylin now gives a rich purple color to the cellulose elements which were left unstained by the safranin, thus contrasting sharply with the lignified elements. If cyanin and erythrosin be used, the xylem takes the blue and the cambium and phloëm take the red.

The mere selection of two colors which contrast well is not sufficient. Green and red contrast well, but safranin and iodine

green would be a poor combination, for both would stain chromosomes, and neither would stain the spindle; both would stain lignified structures and neither would give satisfactory results with cellulose walls. Both stains are basic. Acid green would have given a contrast in both these cases, because it stains achromatic structures and cellulose walls. In general, an acid stain should be combined with a basic one, but there are so many exceptions that it is hardly worth while to learn a list of basic and acid stains. Stains which stain chromosomes are likely to be basic, and those which do not stain chromosomes are likely to be acid or neutral. If it were true that acid stains affect only basic structures, and basic stains affect only acid structures, a classification of stains would be of great value. Safranin and gentian-violet are both basic, but with proper washing out the chromosomes are red and the spindle is violet, the safranin being washed out from the spindle, while the gentian-violet is washed out from the chromosomes. The only way to insure success is to become familiar with the action of each stain upon the various structures.

THEORIES OF STAINING

In 1890 Auerbach, a zoölogist, published the results of his studies upon spermatozoa and ova. He found that, if preparations containing both spermatozoa and ova were stained with cyanin and erythrosin, the nuclei of the spermatozoa took the cyanin, while the nuclei of ova preferred the erythrosin; hence he proposed the terms "cyanophilous" and "erythrophilous." Auerbach regarded these differences as an indication of sexual differences in the cells.

Rosen (1892) supported this theory, and even went so far as to regard the tube nucleus of the pollen grain as female, on account of its erythrophilous staining. In connection with this theory it was suggested that the ordinary vegetative nuclei are hermaphrodite, and that in the formation of a female germ nucleus the male elements are extruded, leaving only the erythrophilous female elements; and, similarly, in the formation of a male nucleus the female elements are extruded, leaving only the cyanophilous male elements.

As long ago as 1884 Strasburger discovered that with a mixture of fuchsin and iodine green the generative nucleus of a pollen grain stains green, while the tube nucleus stains red. In 1892[1] he discussed quite thoroughly the staining reactions of the nuclei. The nuclei of the small prothallial cells of gymnosperm microspores are cyanophilous like the male generative nuclei. The nuclei of a nucellus surrounding an embryo-sac are also cyanophilous, while the nuclei of structures within the sac are erythrophilous. His conclusion is that the cyanophilous condition in both cases is due to poor nutrition, while the erythrophilous condition is due to abundant nutrition. A further fact in support of the theory is that the nuclei of the adventitious embryos which come from the nucellus of *Funkia ovata* are decidedly erythrophilous, while the nuclei of the nucellus to which they owe their food-supply are cyanophilous.

In division stages nuclei are cyanophilous, but from anaphase to resting stage the cyanophilous condition becomes less and less pronounced, and may even gradually change to the erythrophilous.

An additional fact in favor of this theory is that in *Ephedra* the tube nucleus which has very little cytoplasm about it is cyanophilous. Strasburger claims that there is no essential difference between male and female generative nuclei, and subsequent observation has shown that within the oöspore the sex nuclei are alike in their reaction to stains.

Malfatti (1891) and Lilienfeld (1892–3) claim that these reactions are dependent upon the amount of nucleic acid present in the structures. During mitosis the chromosomes consist of nearly pure nucleic acid and are intensely cyanophilous, but the protoplasm, which has little or no nucleic acid, is erythrophilous. There is a gradual transition from the cyanophilous condition to the erythrophilous, and *vice versa*, the acid structures taking basic stains and basic structures the acid stains.

The terms "erythrophilous" and "cyanophilous" have fallen into disuse, since the affinity is for basic and acid dyes, rather than for blue or red colors. That the terms are misnomers

[1] *Verhalten des Pollens.*

becomes evident when a combination like safranin (basic) and acid green (acid) is used, for the cyanophilous structures stain red, and the erythrophilous green.

Probably but few investigators who have attained any proficiency in microtechnique have not asked themselves how much dependence can be placed upon staining reactions as a means of analysis. Do staining reactions enable us to determine the chemical composition of a structure? If two structures stain alike with Delafield's hæmatoxylin, does this mean that they have the same chemical composition; or if, on the other hand, they stain differently, must they necessarily be different in their chemical composition? Delafield's hæmatoxylin, when carefully used, gives a rich purple color, but a careful examination will often show that in the same preparation some structures stain purple, while others stain red. Does this mean that the purple and red structures must have a different chemical composition? Many people believe that structures which stain differently with a given stain must be chemically different, but they readily agree that structures which stain alike are not necessarily similar in chemical composition. Chromosomes of dividing nuclei and lignified cell walls stain alike with safranin; chromosomes and cellulose cell walls stain much alike with Delafield's hæmatoxylin; but everyone recognizes that the chromosome is very different in its chemical composition from either the cellulose or the lignified wall.

According to Fischer (1897 and 1900), stains indicate physical but not chemical composition. Fischer experimented with substances of known chemical composition. Egg albumin was shaken until small granules were secured. These were fixed with the usual fixing agents, and then stained with Delafield's hæmatoxylin. The extremely small granules stained red, while the larger ones became purple. Since the granules are all alike in chemical composition, Fischer concluded that the difference in staining must be due to physical differences. With safranin, followed by gentian-violet, the larger granules stain red and the smaller violet; if, however, the gentian-violet be used first, then treated with acid alcohol and followed by safranin, the larger

granules take the red and the smaller the gentian-violet. In root-tips similar results were obtained. Safranin followed by gentian-violet stained chromosomes red and spindle fibers violet, while gentian-violet followed by safranin stained the chromosomes violet and the spindle red. One often reads that chromosomes owe their strong staining capacity to nuclein, and especially to the phosphorous, but, according to Fischer, this is shown to be unfounded, since albumin gives similar results, yet contains no phosphorous, and is not chemically allied to nuclein. Delafield's hæmatoxylin is one of the so-called nuclear stains. The nuclei of animals and plants stain deeply with this reagent, but cellulose membranes, the dense protoplasm of embryonic cells, the pyrenoids of green algæ, and many other structures resemble nuclei in their staining. The most critical work on this subject has been done by those who are investigating the structure of the Cyanophyceae and Bacteria to determine whether these forms have nuclei or not. Bütchli claims that the granules which stain red with hæmatoxylin are to be identified with chromatin, while Fischer, whose results have just been given, claims that staining indicates merely physical differences. The subject cannot yet be regarded as settled, but whatever may be true in regard to these conflicting theories, all agree that stains are of the highest importance in differentiating structures, and in bringing out details which would otherwise be invisible.

PRACTICAL HINTS ON STAINING

The number of stains in the catalogues is becoming so great that it is impossible to become proficient in the use of all of them. It is far better to master a few of the most valuable stains than to do indifferent work with many. The beginner, especially if rather unacquainted with the details of plant structure, may believe that he has an excellent preparation when it is really a bad, or at most an indifferent, one. To illustrate, let us suppose that a pollen mother-cell in a late spirem stage has been stained with cyanin and erythrosin. A preparation in which the cell merely shows a differentiation into nucleus and

cytoplasm must be classed as bad; if the nucleus shows a definitely outlined spirem thread, the preparation is better, but is still only indifferent; if the thread appears as a delicate red ribbon bordered by blue granules, the staining may be regarded as a success. If mitotic figures have been stained with cyanin and erythrosin, a first-class preparation should show blue chromosomes and red spindles; if stained with safranin and gentian-violet, the chromosomes should be red and the spindles violet.

In staining growing points, apical cells, young embryos, antheridia, archegonia, and many such things, the cell walls are the principal things to be differentiated, if the preparations are for morphological study. As a rule, it is better in such cases not to use double staining, but to select a stain which stains the cell walls deeply without obscuring them by staining starch, chlorophyll, and other cell contents. For example, try the growing point of *Equisetum*. The protoplasm of such growing points is very dense. If Delafield's hæmatoxylin and erythrosin be used, the hæmatoxylin will stain the walls and nuclei, and will slightly affect the other cell contents, but the erythrosin will give the cytoplasm such a dense stain that the cell walls will be seriously obscured. It would be better to use hæmatoxylin alone. For counting chromosomes, it is better to stain in iron alum-hæmatoxylin alone, or in safranin alone. The same suggestion may well be observed in tracing the development of antheridia, archegonia, embryos, and similar structures.

Permanent preparations are an absolute necessity for the greater part of most advanced work, but let us not imagine that we cannot examine anything until we have made a permanent mount. It would be impossible to make a permanent mount of the rotation of protoplasm. It is better for many purposes to look at motile spores while they are moving. Use *Spirogyra* while it is fresh and green, and use permanent preparations only to bring out nuclei and other details which are not so easily seen in living material. Examples might be multiplied.

CHAPTER V

TEMPORARY MOUNTS AND MICROCHEMICAL TESTS

Before considering the complicated methods involved in making permanent preparations, one should acquire some facility in making mounts for immediate use. The student who fancies that he cannot examine a structure until he has a carefully stained microtome section, will not make much progress in modern botany. That particular class of temporary mounts intended only for chemical tests is considered separately in the second part of this chapter.

TEMPORARY MOUNTS

A preliminary examination of almost any botanical material may be made without any fixing, imbedding, or staining. If a little starch be scraped from a potato, and a small drop of water and a cover-glass be added, a very good view will be obtained, and if a small drop of iodine solution be allowed to run under the cover, the preparation, while it lasts, is better than some permanent mounts. The unicellular and filamentous algæ can be studied quite satisfactorily from such mounts. The protonema of mosses and the prothallia of ferns should be studied in this way, even if a later study from sections is intended. The addition of a little iodine identifies the starch and makes the nucleus more plainly visible. If the top of a moss capsule be cut off at the level of the annulus, a beautiful view of the peristome may be obtained by simply mounting in a drop of water, or, in a case like this where no collapse is to be anticipated, the object may be mounted in a small drop of glycerine—just enough to come to the edge of the cover without oozing out beyond—and the preparation made permanent by sealing with gold size or any good cement. The antheridia and archegonia of mosses may be examined if the surrounding leaves are carefully teased away with needles. Free-hand sectioning with a sharp razor and judicious

teasing with a pair of needles will give a fair insight into the anatomy of the higher plants without demanding any further knowledge of technique. This rough work is a very desirable antecedent to the study of microtome sections, because most students see in a series of microtome sections *only* a series of sections when, in the mind's eye, they ought to see the object building itself up in length, breadth, and thickness as they pass from one section to another.

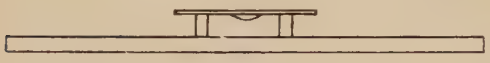

Fig. 11

The movements of protoplasm can, of course, be studied only in the living material. Every laboratory should keep *Chara* growing at every season of the year. Mount a small portion and note the movements in the internodal cells. Avoid any pressure and any lowering of the temperature. A gentle raising of the temperature will accelerate the movements. *Elodea* shows the movements very clearly, especially in the midrib region. The stamen hairs of *Tradescantia* have long been used, their color, resembling a faint hæmatoxylin stain, making them particularly favorable. Stinging hairs show a brisk movement if they are mounted quickly and without injury. Fortunately, the common onion always furnishes favorable material for demonstrating the movements of protoplasm. Strip the epidermis from one of the inner scales of the bulb and mount in water. The granules may appear to better advantage in yellow light, like that of an ordinary kerosene lamp.

The germination of spores and the growth of pollen tubes can be studied in the hanging drop. For facilitating such cultures there are many devices, such as hollow-ground slides, glass rings, rubber rings, etc. (Fig. 11).

Fig. 12

A device which is better for most purposes, and which is easily made by any student is shown in Fig. 12.

A square or round hole $\frac{5}{8}$ of an inch in diameter is cut in a

piece of pasteboard $\frac{1}{8}$ of an inch thick, 1 inch wide, and $1\frac{1}{2}$ inches long. The pasteboard is then boiled to sterilize it and to make it fit more closely to the slide. While the pasteboard is still wet, press it to the slide, make the culture in a drop of water or culture solution on the cover, and invert the cover over the hole. A little water added at the edge of the pasteboard from time to time will keep it from warping and will at the same time provide a constant moist chamber.

In collecting material for mitotic figures in anthers, it is necessary to examine fresh anthers, if one wishes to avoid a tedious and uncertain search after the anthers have been imbedded. By teasing out a few cells from the apex and a few from the base of the anther, the stage of development is readily determined, and anthers which do not show the desired stages can be rejected. By allowing a drop of eosin or methyl green to run under the cover the figures are more easily detected. The actual progress of mitosis has been observed in living stamen hairs of *Tradescantia.*

MICROCHEMICAL TESTS

Botanical microchemistry has become such a large and such an important subject that it would be impossible to give it an adequate presentation in a book intended for students of morphology. Nearly one-half of Zimmermann's book on microtechnique is devoted to microchemistry. Zimmermann's book is recommended to those who need a full account. We shall give only a few tests, but in considering the various stains we shall indicate the effect of each stain upon the various plant structures.

Starch.—Mount the starch, or starch-containing structures in water, and allow a drop of iodine solution to run under the cover. Starch assumes a characteristic blue color. The solution may be prepared by dissolving 1 g. of potassium iodide in 100 c.c. of water and adding 0.3 g. of sublimed iodine. A strong solution of iodine in alcohol (about 1 g. in 50 c.c. of absolute alcohol) keeps well. A drop of this solution added to 1 c.c. of water is

good for testing. With too strong a solution, the starch first turns blue but rapidly becomes black.

Grape Sugar.—In cells containing grape sugar, bright red granules of cuprous oxide are precipitated by Fehling's solution. It is better to keep the three ingredients in separate bottles, because the solution does not keep long after they are mixed. The solutions may be labeled A, B, and C.

A	Cupric sulphate	3 g.
	Water	100 c.c.
B	Sodium potassium tartrate (Rochelle salt)	16 g.
	Water	100 c.c.
C	Caustic soda	12 g.
	Water	100 c.c.

When needed for use, add to 10 c.c. of water 5 c.c. from each of the three solutions. The sections, which should be two or three cells in thickness, are warmed in the solution until little bubbles are formed. Too much heat must be avoided. Mount and examine in a few drops of the solution. The twig or organ may be treated with the solution, and the sections may be cut afterward. Other substances precipitate copper, and may be mistaken for grape sugar by the beginner.

Cane Sugar.—Cuprous oxide is not precipitated from Fehling's solution by cane sugar, but after continued boiling in this solution the cane sugar is changed to invert-sugar and the copper is precipitated. The solution becomes blue.

Proteids.—The proteids turn yellow or brown with the iodine solution. It is better to use a stronger solution than when testing for starch. It must be remembered that many other substances also turn brown when treated with iodine.

When proteids are warmed gently in concentrated nitric acid, the acid becomes yellow. The color may be deepened by the addition of a little ammonia or caustic potash.

When proteids are heated with Millon's reagent, the solution becomes brick-red or rose-red. This reaction takes place slowly even in the cold. The following is one formula for this reagent:

Mercury	1 c.c.
Concentrated nitric acid	9 c.c.
Water	10 c.c.

Dissolve the mercury in the nitric acid and add the water.

Fats and Oils.—The fatty oils are not soluble in water and are only slightly soluble in ordinary alcohol. They dissolve readily in chloroform, ether, carbon disulphide, or methyl alcohol.

Alcannin colors oils and fats deep red. The test is not decisive, because ethereal oils and resins take the same red color. Dissolve commercial alcannin in absolute alcohol, add an equal volume of water, and filter. The fats and oils in sections left in this solution for 24 hours should be bright red. The reaction is hastened by gentle heating.

Osmic acid, as used in fixing agents, colors fats and oils brown or black. The dark color is removed by bleaching in a 3–10 per cent. solution of hydrogen peroxide.

Cellulose.—In concentrated sulphuric acid cellulose swells and finally dissolves. It is also soluble in cuprammonia. The cuprammonia can be prepared by pouring 15 per cent. ammonia water upon copper turnings or filings. Let the solution stand in an open bottle. It does not keep well, but its efficiency is readily tested. Cotton dissolves almost immediately as long as the solution is fit for use.

With iodine and sulphuric acid cellulose turns blue. Treat first with the undiluted iodine potassium iodide solution described in the test for starch, then add a mixture of two parts of concentrated sulphuric acid and one part of water.

With chloroïodide of zinc cellulose turns violet. Dissolve commercial chloroïodide of zinc in about its own weight of water and add enough metallic iodine to give the solution a deep brown color.

Lignin.—Lignified walls are insoluble in cuprammonia. The iodine and sulphuric acid or the chloroïodide of zinc, used as in testing for cellulose, give the lignified walls a yellow or brown color. After a treatment with Schultze's maceration fluid, lignified membranes react like cellulose.

Phloroglucin in a 5 per cent. aqueous or alcoholic solution applied simultaneously with hydrochloric acid, gives lignified walls a reddish-violet color. The preparations do not keep.

Cutinized and Suberized Walls.—These are insoluble in cuprammonia or concentrated sulphuric acid. They are colored yellow or brown by chloroïodide of zinc, or by iodine and sulphuric acid, when applied as in testing for cellulose or lignin. With alcannin, they take a red color, but the red is not as deep as in case of fats and oils. After soaking in an aqueous solution of caustic potash, suberized membranes take a red-violet color when treated with chloroïodide of zinc.

Gum, Mucilage, and Gelatinized Membranes.—These are all soluble in water and are further characterized by their strong power of swelling. They are insoluble in alcohol. A series of forms with various color reactions is included under this heading.

Crystals.—Nearly all crystals which are found in plants consist of calcium oxalate. Crystals of calcium carbonate, calcium tartrate, and calcium sulphate also occur. Calcium oxalate is soluble in hydrochloric acid or nitric acid. It is better to use the concentrated acids. The crystals are insoluble in water and acetic acid. Sulphuric acid changes calcium oxalate into calcium sulphate. When treated with barium chloride, crystals of calcium sulphate become covered with a granular layer of barium sulphate, while crystals of calcium oxalate are not affected.

Calcium carbonate, when treated with hydrochloric acid or acetic acid, dissolves with effervescence. The acetic acid should be rather dilute.

CHAPTER VI

FREE-HAND SECTIONS

This chapter deals not only with free-hand sections, but with microtome sections which may be cut without imbedding. Other objects which may be treated like such sections are also considered here.

The beginner is advised to start with the free-hand section, because the processes are rapid, and it is comparatively easy to find the causes of imperfections and failures. In the paraffin method, where the processes are more complicated, it is often difficult, or even impossible, to determine the exact cause of a failure.

As a matter of fact, real free-hand sections, cut by holding the object in one hand and the knife in the other, are becoming less and less frequent in well-equipped laboratories. A sharp razor is a necessity. For cutting sections of twigs, roots, rhizomes, and similar objects, a razor like the one shown in Fig. 5, *A*, should be used; while for sections of soft tissues, like young asparagus stems, young ovaries of plants, most leaves, and such things, the type of razor shown in Fig. 5, *B*, is much better. In cutting, brace the forearms against the sides, hold the object firmly in the left hand, and cut with a long, oblique stroke from left to right. The edge of the razor and the direction of the stroke should be toward the body, not away from it as in whittling. If the material is fresh, the object and the razor should be kept wet with water, the razor being dipped in water for every stroke. For hard objects, like twigs of oak or maple, the razor will need sharpening after cutting a dozen sections. It is a waste of time to put off sharpening until the razor has become noticeably dull, for all sections except those cut when the razor is perfectly sharp are sure to be inferior. With softer material the razor may hold its edge for hundreds of sections. Those sections which seem to be worth further treatment should be placed at once in water or a fixing agent.

The hand microtome (Fig. 2) makes it possible for even the awkward student to secure fairly good sections. Clamp the object in the microtome, grasp the microtome firmly in the left hand or fasten it to the table, and then cut with a long, even stroke, using the full length of the cutting edge. If the microtome has been fastened to the table, it is unimportant whether the stroke be toward or away from the body.

The sliding microtome (Fig. 3) reduces to a minimum the necessity for manual dexterity. The razor or microtome knife should be set obliquely so as to use as much as possible of the cutting edge. Always remember to keep the knife and object wet. In cutting preserved material, the object and the knife may be kept wet with the preservative.

WOODY AND HERBACEOUS SECTIONS

Safranin and Delafield's Hæmatoxylin.—In order to make the directions as explicit as possible, let us follow the processes from collecting the material to labeling the slide. The rhizome of *Pteris aquilina* is a good object to begin with. Dig down carefully until the rhizome is exposed; then with a sharp knife cut off pieces a few inches in length, taking the greatest care not to strain the tissues. If the rhizome has been cut carelessly or pulled up, as is usually the case, the finished mount will show ruptures between the bundles and bundle sheaths, making your work look like the preparations sold by optical companies.

While the material is still fresh and moist, cut the sections and place them at once into 95 per cent. alcohol, where they should remain 5–15 minutes. It is not necessary to use a large quantity of alcohol; 10 c.c. is enough for 100 thin sections of the rhizome.

Pour off the alcohol and pour on an alcoholic solution of safranin (a 1 per cent. solution of safranin in 50 per cent. alcohol. See chapter on "Formulæ"). It is better to let the safranin act over night, or even for 24 hours.

Pour off the safranin (which may be used repeatedly) and pour on 50 per cent. alcohol. The alcohol will gradually wash out the safranin, but this stain is washed out more rapidly from

cellulose walls than from those which are lignified. The sections should be examined frequently until the stain has been washed out from the cellulose walls, but still shows a brilliant red in the large lignified tracheids. If this differentiation is not secured in 5 or 10 minutes, a drop of hydrochloric acid added to the alcohol will hasten the process. Sometimes a good stain may be secured in 20 or 30 minutes, but the washing-out process is likely to be rapid and uncertain.

Pour off the alcohol and wash the sections thoroughly in ordinary drinking-water. The washing should be particularly thorough if acid has been used to hasten the previous process, for the preparations will fade if any acid remains.

Stain in Delafield's hæmatoxylin 1–30 minutes. Usually 5 minutes will be about right. Delafield's hæmatoxylin will stain the cellulose walls, but will have little or no effect upon lignified structures.

Wash in drinking-water for about 3 minutes. If the cellulose walls show only a faint purplish color, put the sections back into the stain and try a longer period. If the color is a deep purple or nearly black, add a little hydrochloric acid (one drop to 50 c.c. is enongh) to the water. It is better to put the drop into a bottle of water and shake thoroughly before letting the acidified water act upon the sections. As soon as the sections begin to appear reddish, which may be within 2 or 3 seconds, pour off the acidified water and wash in drinking-water, changing the water three or four times a minute, until the reddish color caused by the acid has been replaced by the rich purple color so characteristic of hæmatoxylin. The acid not only secures differentiation by dissolving out the stain from lignified structures more rapidly than from cellulose walls, but it also removes the disfiguring precipitates which almost invariably accompany staining with Delafield's hæmatoxylin. After the purple color has appeared, the sections should be left in water for 20 or 30 minutes. They might be left for several hours.

Now place the sections in 95 per cent. alcohol for about half a minute, and then in 100 per cent. alcohol for 2 or 3 minutes.

The 95 per cent. alcohol is merely to save the more expensive absolute alcohol, which would be weakened very rapidly by the water brought in with the sections.

Pour off the alcohol (which may be used three or four times), and use filter paper to remove every drop which can be reached. Then pour on clove oil. This should clear the sections in a few seconds, or, at most, in 1 or 2 minutes. Instead of clove oil, xylol may be used.

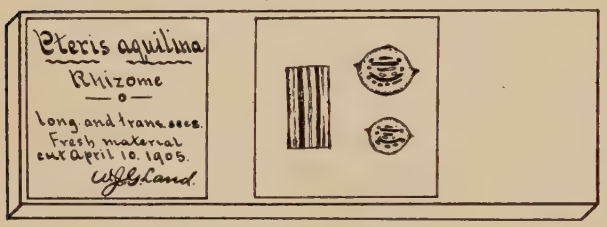

FIG. 13

With a section lifter, or scalpel, or brush, but never with forceps, transfer three or four sections to a clean, dry slide, put on one or two drops of balsam, and add a cover, first heating it gently to remove moisture. If xylol has been used for clearing, it is necessary to work rapidly; for the sections must never be allowed to dry. Use square or oblong covers for such mounts, reserving round covers for glycerine mounts. If material is abundant, use as many sections as you can cover conveniently. If you have used several stains with the same material, select for each mount sections from the different stains. In ordinary wood sections each mount should show the three most important views, transverse, longitudinal radial, and longitudinal tangential sections. It is wasteful to use three slides and three covers to show these three views, or to make a mount containing only a single section of the rhizome of *Pteris*.

Put the label at the left. Write first the genus and species; then indicate what part of the plant has been mounted. The date on which the material was fixed is often valuable. The date of making the mount is of little importance. The beginner is likely to write also the stains used, and other details, which he will find quite unnecessary after a little experience. Fig. 13 illustrates a good style of labeling and mounting.

The following is a convenient summary of the foregoing processes, beginning with the sections in water:

1. Sections in water.
2. 95 per cent. alcohol, 5–15 minutes.
3. Safranin, 1–24 hours.
4. 50 per cent. alcohol (acidified if necessary), 2–15 minutes.
5. Water, 1–5 minutes.
6. Delafield's hæmatoxylin, 1–30 minutes.
7. Water (acidified if necessary), 3 minutes; must be followed by pure water if acid has been used.
8. 95 per cent. alcohol, about half a minute.
9. Absolute alcohol, 2–3 minutes.
10. Clove oil, 1–2 minutes.
11. Balsam.
12. Cover and label.

Since it usually happens that processes are commenced, but cannot be completed at a single laboratory period, it is necessary to know where sections may be left for several hours or until the next day without suffering injury. At 3, 5, or the pure water of 7 in the above schedule, sections may be left until the next day. If it is not desirable to mount all of the sections which have been prepared, they may be kept indefinitely in clove oil or xylol. If the sections are to remain for a year or more in the clearing agent, xylol is to be preferred. Shells with good corks are best for keeping such material.

Iodine Green and Acid Fuchsin is another good combination for such sections. The stain will be particularly brilliant if sections from fresh material are fixed in 1 per cent. chromo-acetic acid for 10–24 hours; and then washed for an hour in water. Beginning with the sections in water, the procedure is as follows:

Stain in aqueous iodine green for 12–24 hours. Then wash in water until the stain is nearly all washed out from the cellulose walls, but is still brilliant in the lignified walls. If the stain acts for too short a time, the washing-out process necessary to remove the stain from the cellulose walls will leave only a pale-green color in the lignified walls. Stain in aqueous acid fuchsin for 2–10 minutes. This should stain the cellulose walls sharply, but

should not act long enough to affect the lignified tissues. Pour off the stain (which may be used repeatedly), and pour on 95 per cent. alcohol, and immediately pour it off and add absolute alcohol. The 95 per cent. alcohol should not act for more than 5 or 10 seconds, its only function being to save the more expensive absolute alcohol. From 10–30 seconds will usually be long enough for the absolute alcohol. Too long a period in the alcohols will weaken the stain. Clear in xylol or clove oil, and mount in balsam.

If a 50 or 70 per cent. alcoholic solution of iodine green has been used, the stain should be washed out in 50 per cent. alcohol; otherwise the treatment is the same.

Methyl Green (aqueous solution) and acid fuchsin is a good combination, and the student may find it easier to get a good differentiation than with iodine green. Follow the directions for the aqueous iodine green and acid fuchsin. It may be necessary to wash more rapidly, since the methyl green is easily extracted.

Other Combinations might be suggested, e. g., iodine green or methyl green with Bismarck brown, methyl green with Delafield's hæmatoxylin, orange G might be added after the safranin and Delafield's hæmatoxylin, and various other stains might be tried. In double staining it is usually best to combine a basic with an acid stain. Green and red make a good contrast, but a section stained with iodine green and safranin would be a failure, because both stains would stain the xylem and neither would stain the cellulose. Both stains are basic. Red lignin and green cellulose could be secured by using safranin and acid green. Green lignin and red cellulose, as already indicated, can be got with iodine green and acid fuchsin.

The Time Required for the different processes varies greatly, and the time required for a subsequent process is often more or less dependent upon the time given to processes which preceded it. Good mounts of sections of the petiole of *Nuphar advena* have been secured from material which had been cut, fixed, stained in safranin and Delafield's hæmatoxylin, and mounted in balsam, the entire time being less than 30 minutes. This is an

extreme case, and nothing is gained, except time, and the saving of time is apparent rather than real, for the histologist always has something to do while sections are in the stain.

Preserved Material.—If sections are to be cut from material preserved in formalin, the piece should be washed in water, since the odor is annoying and the fumes are injurious to the eyes.

The sections are placed in the stain from water. Sections from alcoholic material are transferred directly to the stain. If the material is in a mixture of alcohol and glycerine, the sections should be washed in water or 50 per cent. alcohol until the glycerine has been removed before transferring to the stain.

Use fresh material whenever possible, for stains are much more brilliant with fresh material than with material which has lain for a long time in some preservative.

OBJECTS MOUNTED WITHOUT SECTIONING

Fern Prothallia, mounted without sectioning, make very useful preparations. Select desirable stages and fix in chromo-acetic acid for 10–24 hours; wash in water for 3 or 4 hours, changing the water frequently; stain in Delafield's hæmatoxylin for 5–30 minutes; wash in slightly acidulated water for a few seconds, and then wash thoroughly in pure water. The prothallia must now be brought through a graded series of alcohols, 15, 35, 50, 70, 85, 95, and 100 per cent. being sufficiently close to prevent plasmolysis. Then use mixtures of alcohol and xylol, 3 parts absolute alcohol and 1 part xylol, 2 parts alcohol and 2 parts xylol, 1 part alcohol and 3 parts xylol, and then pure xylol. Then bring the sections into a mixture of xylol and balsam, using at least 10 parts of xylol to 1 of balsam. If left in a shell, without corking, the xylol will soon evaporate, so that in a few days the prothallia may be mounted. Use the balsam in which the material has been standing, because any other balsam may have a different concentration. At every step in the process the prothallia should be examined under a microscope, so that any plasmolysis may be detected. If each succeeding step is tested with a single prothallium, a general disaster may be avoided. If

plasmolysis takes place weaken the reagent and try another prothallium. When a safe strength is found, bring on the bulk of the material, and use the same method with succeeding steps. The dangerous places are likely to be the transfer from alcohol to xylol and the transfer from xylol to balsam. The process is tedious, but the mounts are very firm and durable. Mounts in glycerine or glycerine jelly are quickly made, and are just as good, except that they must be handled more carefully.

Sori of Ferns.—Instructive mounts of sori or of individual sporangia may be made without sectioning. It is better to choose ferns with thin leaves, since leaves thicker than those of *Asplenium thelypteroides* are likely to be unsatisfactory. If this fern is at hand, cut off several of the small lobes which bear three to six pairs of sori. Fix in chromo-acetic acid; wash in water; stain in Delafield's hæmatoxylin, or omit staining altogether; pass through a series of alcohols, allowing each grade to act for at least 10 minutes; clear in clove oil; and mount in balsam. If the sori have begun to turn brown, better views of the annulus will be obtained without staining.

Mosses and Liverworts.—Nearly all mounts are more successful by other methods, for which the student should consult the chapter on Bryophytes. Excellent mounts of the peristome of the moss can be made as follows: From fresh or preserved capsules cut off the peristome just below the annulus. Treat with 95 per cent. alcohol 1 minute, absolute alcohol 2–5 minutes, clear in clove oil or xylol, and mount in balsam. It is a good plan to put at least three peristomes on a slide, one with the outside up, one with the inside up, and another dissected to show details of the teeth.

Fairly good unstained mounts of the archegonia and antheridia of small mosses can be obtained by following the directions for mounting the sori of ferns.

Beautiful and instructive mounts of the more delicate foliose Jungermanniaceae can be made by staining lightly in Delafield's hæmatoxylin whole plants, or pieces as long as can be covered conveniently. The method is that just given for fern prothallia. The mount should show both dorsal and ventral views.

The Epidermis shows its best surface views without sectioning. Select some form with large stomata, like *Lilium* or *Tulipa*, strip pieces of epidermis from both sides of the leaf, and place them immediately in absolute alcohol for 1 or 2 minutes. Stain in Delafield's hæmatoxylin; after this stain is satisfactory and all acid has been washed out, stain for 1 or 2 minutes in aqueous eosin, erythrosin, or acid fuchsin; place directly into 95 per cent. alcohol for a few seconds (merely to save the absolute alcohol), then into absolute alcohol for about 30 seconds, and then into clove oil. Mount in balsam. The epidermis is likely to curl and, unfortunately, patience seems to be the only remedy. In mounting, be careful to get pieces from both sides of the leaf, and be sure that the pieces are outside up. The inside of the epidermis is usually more or less rough, on account of the mesophyll torn off with it.

Other Objects.—The cases just given will suggest other objects which might be mounted by this method. In Part II of this book, this method is frequently recommended for mounting certain structures. Aside from the tediousness of the method, the principal objection to it is the danger from plasmolysis. Were it not for these objections, the method would replace the glycerine method, except when glycerine preserves the natural color of an object. The Venetian turpentine method will doubtless come into general use, not only for filamentous algæ and fungi, but also for mounting objects like entire fern prothallia.

CHAPTER VII

THE GLYCERINE METHOD

Unicellular forms and the filamentous algæ and fungi are usually mounted in glycerine or in glycerine jelly. The method is simple and easily mastered, but for most forms is inferior to the Venetian turpentine method. Glycerine preserves, to a considerable extent, the green color of chlorophyll, a great advantage in mounts of moss protonema and similar objects where it is desirable to retain the natural color. Filamentous forms do not become brittle, but may be arranged with needles without much danger. The method, as used in connection with staining and without staining, will be described.

Haidenhain's Iron-Hæmatoxylin.—A good form to begin with is the familiar *Spirogyra*. Select as large a species as is available. Fix in chromo-acetic acid for 10–24 hours. The strength of the fixing agent must be determined for every collection of material. If there is in the laboratory a stock solution of 1 per cent. chromo-acetic acid (1 g. chromic acid and 1 c.c. glacial acetic acid to 100 c.c. of water), take one part of this stock solution and add an equal amount of water; then add 1 c.c. of glacial acetic acid to each 100 c.c. of the weakened solution. Place a few filaments in the solution, and if plasmolysis occurs, weaken the solution by adding water, and then try again. When a solution is found which causes no shrinkage, fix the material for 10–24 hours. Wash in water for 2–4 hours, changing the water frequently.

Put a few filaments into a 3 per cent. solution of ammonia sulphate of iron. If plasmolysis occurs, weaken the solution by adding water. When the proper strength has been determined, put the material into the mordant for 1 or 2 hours. Wash in water for 5–30 minutes, and then stain in $\frac{1}{2}$ per cent. aqueous solution of hæmatoxylin for 2–24 hours. Wash again in water

for 5–30 minutes, and then place the material for a second time in the iron solution. The material must now be examined every few minutes, since the iron solution extracts the stain. When the stain is just right, wash in water for 1–4 hours. If the iron solution is not washed out thoroughly, its continued action will cause the preparations to fade.

Put the material into 10 per cent. glycerine (1 part glycerine and 9 parts water), and then allow the water to evaporate gradually in a place as free from dust as possible. Minots, or watch crystals, are good dishes for this purpose. The white glass covers of "Hazel" jars could hardly be surpassed. Petri dishes are also good, but rather expensive. When the glycerine has become about as thick as pure glycerine, the material is ready for mounting. A little to the right of the center of the slide, place a drop of the glycerine in which the material is lying. In the drop place a little of the material, taking care not to use more than can be spread out without making a confusing tangle. Use scissors constantly so as not to injure filaments by trying to pull them out from a tangle. There should be *just enough* glycerine to come to the edge of the cover-glass, but *not any more*, for it is impossible to seal a mount if glycerine has oozed out beyond the cover.

The mount should now be sealed. Canada balsam, various asphalts, cements, and glues have been used, but the best and cheapest of all seems to be the ordinary flat varnish, or gold size, used by painters in laying gold leaf. Choose a gold size of about the color of the varnish used for ordinary woodwork. Mounts which had been sealed with gold size more than fifty years before have been exhibited in perfect condition. The gold size, as painters use it, is likely to be too thin for sealing mounts. Put some of it in a one-ounce bottle with a wide neck and leave the cork out until the gold size thickens a little. Should it become too thick, thin it with turpentine.

Nothing but practice will enable one to spin a good ring, but a good camel's-hair brush, a good turntable, and a gold size neither too thick nor too thin will facilitate matters. Give the

turntable a spin, and with the brush touch first the slide about as far out from the cover as you wish the ring to extend, then gradually approach the cover. Dip the brush in the gold size again, and gradually extend the ring until it is about one-sixteenth of an inch wide on the cover. The touch must be extremely gentle or the cover will be moved. Do not try to put on a thick ring the first time, but let a thin ring harden for an hour (months would do no damage), and then a thicker ring can be added without any danger. Thin rings are too likely to be broken, and thick rings are in the way if the preparation is to be examined with high powers. A medium ring is best, and it should consist of two coats, for a crack would seldom appear at the same place in both coats. A good shape and thickness for a ring are shown in Fig. 14.

FIG. 14.—Slide, natural size, showing form and size of the ring.

The following is a summary of the foregoing processes:

1. Fix in chromo-acetic acid, 10–24 hours.
2. Wash in water, 2–4 hours.
3. Iron solution, 1–2 hours.
4. Wash in water, 5–30 minutes.
5. ½ per cent. hæmatoxylin, 2–24 hours.
6. Wash in water, 5–30 minutes.
7. Iron solution until stain is right.
8. Wash in water, 1–4 hours.
9. 10 per cent. glycerine.
10. Mount and seal.

If the material has been fixed in formalin, it should be washed in water for 5–10 minutes before staining. Material preserved in 70 per cent. alcohol should be placed successively in 50 per cent., 35 per cent., 15 per cent. alcohol, and then in water, allowing each to act for 15–30 minutes before being placed in the stain.

Eosin is a good stain for many algæ and fungi, when sharp outlines rather than cell contents are to be brought out. After the material has been fixed and washed in water, stain in an aqueous solution of eosin for 12–24 hours. Wash in water until

the stain is about right. Since subsequent processes will extract a little more of the stain, the washing in water must stop a little before the desired differentiation has been secured. Place in 1 per cent. acetic acid for a few minutes to fix the stain. Then place in 10 per cent. glycerine containing about 1 per cent. acetic acid, and allow the glycerine to concentrate. The acetic acid is to prevent the stain from washing out. When the glycerine has reached the proper concentration, mount and seal as before.

The following is a rapid method for forms like *Eurotium* and *Penicillium:* Fix in 100 per cent. alcohol about 2 minutes; stain in aqueous eosin 5 minutes; wash in water about 1 minute; fix in 1 per cent. acetic acid 1 minute; then mount directly in 50 per cent. glycerine to which about 1 per cent. acetic acid has been added. It is hardly worth while to try this method with forms which have large cells; they are almost sure to collapse. If a form like *Eurotium* passes through the earlier processes without danger, but collapses when put into the 50 per cent. glycerine, put it into the 10 per cent. glycerine and allow the glycerine to concentrate.

Mounting without Fixing or Staining.—It is sometimes desirable to retain the natural color of an object. The chlorophyll green can usually be preserved by mounting directly in glycerine without any previous fixing. Other colors also are often preserved in this way. Moss protonema make beautiful preparations by this method. If possible, select protonema showing the very young moss plants. The brown protonema and brown bulbils preserve their color perfectly. Wash the dirt away from the protonema, which is then placed in 10 per cent. glycerine.

The brown or black spores of fungi are readily mounted in this way.

The method is very useful when one finds a single specimen of *Pediastrum*, or any small form which would be lost in the more complicated processes. Place a large drop of 10 per cent. glycerine on a slide; with a pipette, transfer the object to the drop, and allow the glycerine to concentrate. Then add a cover and seal the mount.

GLYCERINE JELLY

Glycerine jelly is useful for objects which are too large to mount in glycerine without making cells. With objects as large as *Volvox* or branches of *Chara*, the glycerine is likely to ooze out beyond the cover, making it difficult or impossible to seal the mount. Such objects may be mounted in glycerine jelly. The material should be put into 10 per cent. glycerine, which should be allowed to concentrate until it is as thick as pure glycerine. The bottle containing the glycerine jelly is then put into warm water until the jelly melts. No more heat should be applied than is really necessary. Place a drop of the melted jelly on a warm slide, and place on it the material to be mounted. Add a cover, and allow the mount to cool. In cold weather, a glycerine-jelly mount is safe without sealing, but in summer the jelly may melt. It is better to seal all glycerine-jelly mounts.

It is a common practice to put a small piece of the glycerine jelly on the slide and heat the slide until the jelly melts. The only objection is that one may ruin his material by putting it into the drop while it is too hot.

CHAPTER VIII

THE VENETIAN TURPENTINE METHOD

A method, somewhat more tedious and, for the beginner, rather uncertain, but which gives the most beautiful preparations, is described by Pfeiffer and Wellheim.[1] After material has been brought safely into pure glycerine, the glycerine can be washed out with alcohol, and alcoholic stains may be used without much danger from plasmolysis. Mounts are made in Venetian turpentine. The following is a brief description of the most important method given in the long article. In becoming acquainted with the method *Spirogyra* is a good form to work with.

Fix in chromo-acetic acid.

1 per cent. chromic acid . . .	70 c.c.
Glacial acetic acid	5 c.c.
Water	90 c.c.

The volume of the fixing agent should be about 100 times that of the material to be fixed. Usually 12 hours will be long enough; for some algæ, like *Batrachospermum*, 12 hours is too long.

Wash in water for 2–4 hours, and then put the material into 10 per cent. glycerine, and let the water evaporate until the glycerine is as thick as pure glycerine. Wash the glycerine out with 95 per cent. alcohol, and proceed with the staining.

Magdala Red.—This stain is quite satisfactory either when used alone or in combination with other stains. Make a saturated solution in 85 or 90 per cent. alcohol. Mix 1–3 drops of the stain with 20 c.c. of 90 per cent. alcohol, and allow this weak stain to act for 3–6 hours. Pour off the stain and put the material into a 10 per cent. solution of Venetian turpentine (1 part Venetian turpentine and 9 parts absolute alcohol, or 95 per

[1] Pfeiffer, Ferdinand, and Wellheim, R. v., Zur Preparation der Süsswasseralgen *Jahrbücher für wissenschaftliche Botanik*, **26**: 674–732. 1894.

cent. alcohol). It is not necessary to wash out the stain, for the slight amount of stain adhering to the filaments will be taken up during the concentration of the turpentine. It is much better to have the stain too intense than to have it too weak, for overstaining is easily corrected after the mounts have been sealed. Place the preparations on a white background and expose them to bright sunlight, examining them from time to time. When the stain is satisfactory, take care that no further exposure to bright sunlight occurs. Ordinary daylight seems to do no damage, preparations made several years ago being as brilliant as when first finished. Since the direct sunlight does not decolorize all structures with equal rapidity, considerable differentiation may be secured. The stain disappears first from the gelatinous sheath and cell wall, then from the cytoplasm and chromatophore, and finally, if the action be too prolonged, from the nucleus, nucleolus and pyrenoid.

Anilin Blue.—Make a saturated solution in 85 or 90 per cent. alcohol. Even when the dry stain is marked aqueous, it should be made up in strong alcohol. Add a few drops of this solution to 20 c.c. of 90 per cent. alcohol. The addition of a very slight trace of acetic acid is sometimes an advantage. Material comes into the stain from 95 per cent. alcohol, and the staining should continue for 1–3 hours. If the stain is too intense, wash it out with 90 per cent. alcohol. If the washing out should be carried too far, the addition of a trace of acid may restore the stain. A slight overstaining is not objectionable.

Place the material in 10 per cent. Venetian turpentine, and when a sufficient concentration is reached, mount and seal.

Magdala Red and Anilin Blue.—Stain first in Magdala red. Overstaining does no damage. Rinse for a few seconds in 85 per cent. alcohol. Put a few drops of the strong solution of anilin blue into 20 c.c. of 85 per cent. alcohol, and in this weakened mixture stain for 1–10 minutes. The bright-red color will become bluish-violet. Dip the material for 2–5 seconds in acid alcohol (1 drop of hydrochloric acid to 50 c.c. of 85 or 90 per cent. alcohol), and transfer immediately to neutral 95 per

cent. alcohol, which should be changed several times to get rid of the acid. Then put the material into 10 per cent. Venetian turpentine, and proceed as before.

The foregoing paragraphs outline the method essentially as given in the article referred to. The method is practically new in the United States. It may be that the uncertainty in getting good preparations has prevented the method from gaining a wide circulation. We have experimented with the method for several weeks and have tried it with a large class in the laboratory. In this way we have noted where some of the most usual difficulties occur, and, although it is almost a repetition of the preceding paragraphs, we venture to restate the method as we are using it at present.

A RESTATEMENT OF THE METHOD

Using *Spirogyra* as a type, proceed as follows:

1. Fix over night, or for 24 hours in chromo-acetic acid.

Chromic acid	1 g.
Glacial acetic acid	3 c.c.
Water	300 c.c.

2. Wash in water for 2–4 hours.

3. Transfer to 10 per cent. glycerine, and allow the glycerine to concentrate until it has the consistency of pure glycerine. It is not necessary to use an exsiccator. Merely put the glycerine into shallow dishes, and leave it exposed to the air, but protected from dust. If the material is in Petri dishes or other dishes with a large surface, 3 or 4 days will be sufficient. This process should not be hastened by warming.

4. Wash out the glycerine with 95 per cent. alcohol. It will be necessary to change the alcohol several times. Any glycerine left in the material will interfere with staining.

5. Stain in Magdala red. At least two Magdala reds are sold by dealers. The one marked *echt* is more expensive, but, in our experience, is inferior to the one marked simply Magdala red. Make a 1 per cent. solution in 90 per cent alcohol. We use the stain much stronger than recommended by Pfeiffer and Wellheim.

This solution, diluted with an equal volume of 95 per cent. alcohol and allowed to act for 6–24 hours, does not stain too deeply.

Rinse the material for a minute in 90 per cent. alcohol, and then stain in anilin blue, using a 1 per cent. solution in 90 per cent. alcohol, diluted with four times its volume of 90 per cent. alcohol. The time required for successful staining will vary from 3–30 minutes. Do not put all the material into the anilin blue at once, but by trying a few filaments at a time, find out what the probable periods may be.

Rinse off the stain in 90 per cent. alcohol, and then treat for a few seconds in acid alcohol (1 drop of HCl to 30 c.c. of 90 per cent. alcohol). The acid alcohol fixes and brightens the anilin blue, but extracts the Magdala red. If the anilin blue or the acid alcohol act for too short a time, the blue will be weak; if they act too long, the red is lost entirely. If the blue overstains too much, wash it out in 95 per cent. alcohol. If the red overstains, wait until the mount is finished, and then reduce the red by exposing the slide to direct sunlight.

6. Place the material in 10 per cent. Venetian turpentine in a shallow dish, and allow the turpentine to concentrate in an exsiccator. An effective exsiccator is readily made by placing water-free fused calcium chloride in a saucer under a low bell jar, the bell jar resting upon a pane of glass. Half a dozen pieces of the chloride as large as Brazil nuts will last for 2 or 3 weeks. The low dishes containing the material in 10 per cent. Venetian turpentine are placed under the same bell jar. In a large laboratory a tight wooden box two feet long, eighteen inches wide, and six inches deep, resting upon a level table, is perfectly satisfactory. The calcium chloride may be broken up and placed on an oyster plate under the box. The greater the surface of calcium chloride exposed, the more rapidly will the turpentine concentrate. The dishes containing the material in 10 per cent. Venetian turpentine may be placed anywhere under the box. The box is better than the glass bell jar, because the material is kept in the dark. Where a bell jar is used, it should be protected from intense

light. Care must be taken not to let any of the calcium chloride get into the turpentine. The concentration of the turpentine must not be too rapid. Not less than 1 or 2 days should be allowed for the concentration of 30 c.c. of the 10 per cent. turpentine in a watch-glass two inches in diameter. Pfeiffer and Wellheim recommend that the watch-glass, or very short shell, be placed in an air-tight glass jar with some fused calcium chloride. The main point is to secure evaporation of the alcohol without getting any water into the turpentine, which would occur if the watch-glasses should be exposed to the air, as when allowing the 10 per cent. glycerine to concentrate.

If the dishes are sufficiently shallow, like solid watch-glasses, the turpentine will concentrate sufficiently in 1 or 2 days. The turpentine should not become thicker than the balsam used in mounting. If the turpentine should become too thick, thin it by adding Venetian turpentine having the consistency of very thin balsam.

Mount the material in a few drops of the Venetian turpentine and add a cover. Square covers may be used, since it is entirely unnecessary to seal the mounts. Such mounts are as hard and durable as balsam mounts.

The surprising beauty of successful preparations will compensate for the large proportion of failures which are likely to occur. Nuclei and pyrenoids should show a brilliant red, while the chromatophores and cytoplasm should be dark blue. The cell walls should show a faint bluish color.

Material in the thickened Venetian turpentine, if not needed for immediate mounting, may be put into small vials or shells, where it can be kept indefinitely. The shells should be kept out of the light.

Other stains may be used, but they should be dissolved in 85 or 90 per cent. alcohol. If it is desirable to use iron alum-hæmatoxylin or other aqueous stains, the staining should be done after the fixing agent has been washed out in water. The material is then put into 10 per cent. glycerine. When the glycerine has concentrated, it is washed out in 95 per cent.

alcohol, and the material is transferred to 10 per cent. Venetian turpentine, which is allowed to concentrate in the exsiccator.

Fixing agents for other algæ are suggested in Part II, in connection with the various forms.

This method is equally good for filamentous fungi and also for the prothallia of *Equisetum* and ferns, for delicate liverworts and mosses, and similar objects.

CHAPTER IX

THE PARAFFIN METHOD

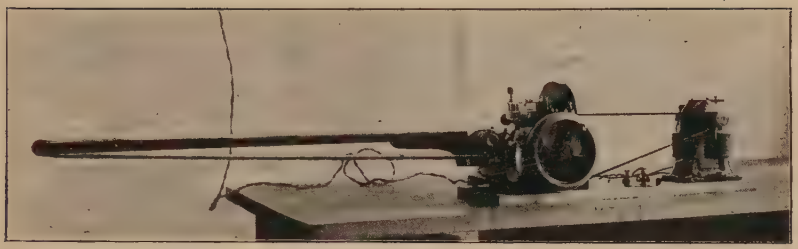

FIG. 15.—Rotary microtome with electric motor attachment, as devised by Dr. W. J. G. Land

The paraffin method is the most important of all histological methods now in use.

The results obtained by this method would have been regarded as almost miraculous by the histologists of one hundred years ago. At that time it was customary to observe things dry, and no cover-glasses were used. Section-cutting with sharp knives or razors did not become general until about 1830. The need for an instrument which would cut sections without demanding an extreme degree of manual dexterity was soon felt, but a successful microtome did not appear until much later. With a thoroughly good modern microtome, series of sections may be cut from an object too small to be seen by the naked eye. One of the most recent innovations in microtome technique is an electric motor attachment. Fig. 15 shows the instrument as planned by Dr. Land. For cutting long ribbons the scheme is very satisfactory.

Many of the principles involved in this method are general in their application, and some of the processes are common to other methods. Before attempting the free-hand sectioning, the beginner should read the following paragraphs on killing and fixing, washing, hardening and dehydrating, and on clearing.

KILLING AND FIXING

As stated in the chapter on "Reagents," the purpose of a killing agent is to bring the life processes to a sudden termination, while a fixing agent is used to fix the cells and their contents in as nearly the living condition as possible. The fixing consists in so hardening the material that the various elements may retain their natural condition during all the processes which are to follow. Usually the same reagent is used for both killing and fixing. Zoölogists, from humane motives, may use chloroform for killing, while other reagents are used for fixing. In fixing root-tips, anthers, and other material for a study of mitotic figures, it is necessary that the killing be very prompt. In a weak solution of chromo-acetic acid, nuclei which have begun to divide may complete the division, although the reagent might hinder nuclei from entering upon division. By treating for 5–20 minutes with Flemming's weaker solution, or with a chromo-acetic solution containing a much smaller proportion of osmic acid, the killing will be greatly accelerated and the proportion of nuclei in division will be correspondingly greater. If filamentous algæ are placed for 1 or 2 minutes in a chromo-acetic solution containing a little osmic acid, all the advantages of immediate killing will be secured. Material is then transferred to chromo-acetic acid containing no osmic acid. The short treatment with an osmic solution is not likely to cause any serious blackening. When the solution containing the osmic acid is allowed to act for only a few minutes, it can be used several times.

Take the killing and fixing fluids into the field. If one waits until the material is brought to the laboratory, there may be some fixing, but it will, in many cases, be too late to do much killing. Material which has begun to wilt is not worth fixing. Material like *Spirogyra*, however, may be brought from the field into the laboratory before fixing, if considerable water be brought with it. Branches with developing buds may be brought in and kept in water. Always have the material in very small pieces, in order that the reagents may act quickly on all parts of the specimens. Pieces larger than one-fourth inch cubes should be avoided when-

ever possible. For very fine work no part of the specimens should require the reagent to penetrate more than one-sixteenth of an inch. In general, the volume of the reagent should be ten to fifty times that of the material. The time required for this process varies with the reagent, the character of the tissue, and the size of the piece. About 24 hours is a commonly recommended period for chromic-acid solutions. While this is longer than is necessary for filamentous algæ and delicate objects which are reached immediately by the fixing agent, no injury would result from a twenty-four hours' treatment. For most embryological material 2 or even 3 three days would probably do no harm.

Directions for making and using the various fixing agents are given in the chapter on "Reagents."

WASHING

Nearly all fixing agents, except the alcohols, must be washed out from the material as completely as possible before any further steps are taken, because some reagents leave annoying precipitates which must be removed, and others interfere with subsequent processes. Aqueous fixing agents with chromic acid as their principal ingredient are washed out with water; aqueous solutions of corrosive sublimate are also washed out with water; but alcoholic solutions should be washed out with alcohol of about the same strength as the fixing agent; picric acid, or fixing agents with picric acid as an ingredient, must not be washed out with water, but with alcohol, whether the picric acid be in aqueous or alcoholic solution. When washing with water, running water is best, and where this is not convenient the water should at least be changed frequently. The washing-out process usually requires from 12 to 24 hours, but it can be shortened about one-half by keeping the water lukewarm.

HARDENING AND DEHYDRATING

After the material has been washed, it is necessary to continue the hardening and also to remove the water. Alcohol is used almost entirely for these purposes. It completes the hardening and at the same time dehydrates, that is, it replaces the water

in the material, an extremely important consideration, for the least trace of moisture is likely to interfere seriously with the infiltration of the paraffin.

The process of hardening and dehydrating must be gradual, if the material should be transferred directly from water to absolute alcohol, the hardening and dehydrating would be brought about in a very short time, but the violent osmosis would cause a ruinous contraction of the more delicate parts. Therefore transfer from water to 15 per cent. alcohol, which should act for 6–24 hours. It is not necessary to use a large amount of the reagent as in fixing and in washing. Enough to cover the material is sufficient. Such alcohols may be used again, but it must be remembered that they are constantly becoming weaker. After being used two or three times the various grades may be poured into a common jar, and the strength of the alcohol can be determined by an alcoholometer. Alcohols of weaker grades may then be made from this "waste alcohol." Pollen grains, fungus spores, starch grains, and various granules are likely to be left in the alcohol, so that, when it is necessary to know the identity of every such structure, only pure alcohols should be used.

Pour off the 15 per cent. alcohol and pour on 35 per cent., which should act for a similar period. In the same way use 50 per cent. and 70 per cent. Material not needed for immediate use can be kept indefinitely in 70 per cent. alcohol. Material left for some time in 70 per cent. alcohol will shrink in spite of good killing and fixing, and its capacity for staining is diminished. Some recommend that glycerine be added to the alcohol; others prefer to complete the dehydrating process and leave the material in an essential oil; while still others would imbed it and keep it in paraffin. The last is doubtless best of all, but requires such an immense amount of labor that it is impracticable for general purposes.

After the 70 per cent. alcohol, use 85 per cent. and 95 per cent., allowing 6–24 hours for each. Then use 100 per cent. alcohol for 1 or 2 days. The 70 per cent. would probably com-

plete all the hardening which is necessary, but the other three must be used to complete the removal of water. Various devices, like constant drips and osmotic apparatus, have been proposed to secure a more gradual transfer. Whether these have any real advantages is very doubtful. Well-fixed fern prothallia may be taken through the series 15 per cent., 35 per cent., 50 per cent., 70 per cent., without the slightest plasmolysis. Such things as fern prothallia, filamentous algæ, etc., can be watched under the microscope as the transfer is made, and, if plasmolysis results, the series of alcohols may be made closer, e. g., 10 per cent., 20 per cent., 30 per cent., etc.

Up to this point the processes are exactly the same, whether the material is to be imbedded in paraffin or celloidin, in both cases the perfect dehydration of the material being necessary.

CLEARING

Let us suppose that the material has been thoroughly dehydrated, so that not the slightest trace of water remains. If the supposition chances to be contrary to fact, all the work which has preceded, as well as all which is to follow, is only an idle waste of time. The purpose of a clearing agent is to make the tissues transparent, but clearing agents also replace the alcohol. At this stage the latter process is the essential one, the clearing which accompanies it being incidental. The clearing, however, is very convenient, since it shows that the alcohol has been replaced and that the material is ready for the next step.

Various clearing agents are in use. Xylol is the most generally employed, and for most purposes it seems to be the best. Bergamot oil, cedar oil, clove oil, turpentine, and chloroform are used for the same purpose. Cedar oil and chloroform may, in some cases, be as good as xylol.

Only a small quantity of the clearing agent is necessary, enough to cover the material being sufficient.

The transfer from absolute alcohol to the clearing agent should be *gradual*, like the hardening and dehydrating processes. The following is a good method:

3 parts 100 per cent. alcohol and 1 part xylol, 1–10 hours.
2 parts 100 per cent. alcohol and 2 parts xylol, 1–10 hours.
1 part 100 per cent. alcohol and 3 parts xylol, 1–10 hours.

These three grades may be kept in three bottles, and may be used repeatedly. It is not a good plan to keep adding xylol to the absolute alcohol without any pouring off, because the pure xylol comes into contact with the material before the absolute alcohol and xylol become mixed. After pouring off the third mixture, add pure xylol, and allow it to act until the material becomes transparent. This may require only a few minutes, but may require hours. Other clearing agents may be used in the same way instead of xylol.

THE TRANSFER FROM CLEARING AGENT TO PARAFFIN

This should also be a *gradual* process. The most convenient method is to place a small block of paraffin in the pure clearing agent with the material. The paraffin dissolves gradually and produces the same result as if a small shaving of paraffin had been added every few minutes for a day or so. During this process the bottle should be kept lukewarm and the cork should never be left out. Six to ten hours, or over night, is usually sufficient for this step, although it would seem that material may be kept here for much longer time without injury, and in case of such refractory material as the megaspores of the heterosporous pteridophytes four or five days seem necessary. Excellent preparations of the embryo-sac of *Aster* have been made from material which had remained in the xylol and paraffin for nearly three years. No more paraffin should be added than will go into perfect solution. The temperature may be gradually increased, so that a much greater amount of paraffin will go into solution, but the paraffin must not be allowed to crystallize.

THE PARAFFIN BATH

This step is usually called infiltration, but when the transfer from the clearing fluid to paraffin is made gradually, as has just

been indicated, the process of infiltration is already begun. It is now necessary to get rid of the xylol or other clearing agent. This may be done by simply pouring off the mixture of xylol and paraffin and replacing it with pure melted paraffin. The cork should now be removed to let the xylol evaporate. The bath should be kept at a temperature about 1° C. higher than the melting-point of the paraffin. Fifty-three degrees C. is a good temperature for general purposes, but this may be reduced from 1° to 3° C. in winter, and must often be raised in summer. For special purposes it is sometimes necessary to use a temperature as high as 70° C. If the xylol or other clearing agent is not thoroughly removed, the paraffin will be granular or mealy, and will not cut well. The paraffin should be changed once or twice to make sure that the clearing agent is all removed. Do not waste this paraffin, for the clearing agent can be driven off by prolonged heating, and the paraffin will be better than ever. Most people use soft paraffin (about 45° C.) for the first half of the time necessary for infiltration, and a harder grade (48° C. to 54° C.) for the latter part of the process. This is a good plan, for soft paraffin melts at a lower temperature, and it is always well to minimize heat.

Some think that it is better not to pour off the mixture of the clearing agent and paraffin, but rather to evaporate the clearing agent by keeping the temperature just high enough to prevent the crystalization of the paraffin. This method has certainly given good results with small, delicate objects.

In large laboratories where it is possible to have several baths, these can be kept at different temperatures, as described on page 7.

The time required for infiltration varies with the character of the tissue and the size of the piece. Few things can be well infiltrated in less than an hour. Lily ovaries require 1–4 hours; heads of *Aster* at the fertilization period, 6–12 hours. Some claim that even delicate objects, like fern prothallia and the filamentous algæ and fungi, are not injured by a bath of several days, if care be taken not to let the temperature rise above

48° C. to 50° C. No one seems to know how long a certain object should remain in the bath, but many competent investigators are now using more prolonged periods.

IMBEDDING

Material may be imbedded in paper trays, Petri dishes, watch crystals, or in apparatus made for the purpose. Imbedding L's consisting of two L-shaped pieces (Fig. 16) of brass, type metal, or lead are very convenient. A pair of L-shaped pieces with arms three inches long will furnish a box of almost any required size. A piece of glass serves for a bottom. The most satisfactory imbedding-dish we have used is a thin rectangular porcelain dish, glazed inside. This dish, called a *Verbrennungsschale*, is made by the Königliche Porzellan-Manufactur, Berlin, Germany. The most convenient sizes are $40 \times 50 \times 10$ mm., $68 \times 45 \times 10$ mm., and $91 \times 58 \times 15$ mm. These are listed respectively at 50, 60, and 80 Pfennige. The tray, Petri dish, or whatever is used, should be slightly smeared with glycerine, to prevent sticking. If several objects are to be imbedded in one dish, it is best to have the dish as near the temperature of melted paraffin as possible; otherwise the objects may stick to the bottom, and it will be impossible to arrange them properly. Hot needles are good for arranging material. Great care should be taken not to have the dish too hot, since too high a temperature not only injures the material, but also prevents a thorough imbedding. Pour the paraffin with the objects into the imbedding-dish and cool as rapidly as possible. If paraffin cools slowly, it crystallizes and does not cut well. The layer of paraffin should be just thick enough to cover the objects, not only as a matter of economy, but because a thick layer retards the cooling. Very small objects, like the megaspores of *Marsilea*, ovules of *Silphium*, etc., may simply be poured out upon a *cool* piece of glass. In this way very thin cakes are made, which harden very rapidly.

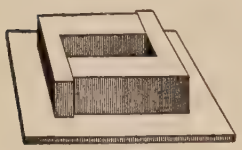

Fig. 16

CUTTING

As soon as the paraffin is thoroughly cooled, it is ready for cutting. Trim the paraffin containing the object into a convenient shape, and fasten it upon a block of wood. Blocks of pine three-fourths of an inch long and three-eighths of an inch square are good for general purposes. Put paraffin on the end of the block so as to form a firm cap about one-eighth of an inch thick. Warm the cap and the bottom of the piece containing the object, and press them lightly together; then touch the joint with a hot needle, put the whole thing into cold water for a minute, and it is ready for

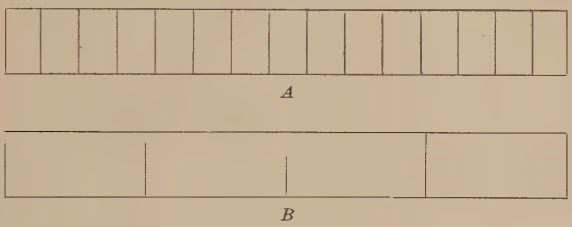

FIG. 17

cutting. Cutting can be learned only by experience, but a few hints may not come amiss: (*a*) Keep the knife *sharp*. If expense is not too serious an objection, it is well to have two hones, one rather soft, for use when the knife is dull, and the other quite hard, for putting on an even edge. Flood the stone with water, and rub it with the small slip which accompanies all high-grade hones; this not only makes a lather which facilitates the sharpening, but it also keeps the surface of the hone flat. As soon as the edge of the knife appears smooth and even under a magnification of thirty or forty diameters, the sharpening is completed with a good strop. It is better to sharpen the knife every time you use it. (*b*) Keep the microtome well oiled and *clean*. (*c*) Trim the block so that each section shall be a *perfect rectangle*.

A ribbon of sections like that shown in Fig. 17, *A* is much better than one like *B* of the same figure, because sections will usually come off in neater ribbons if the knife strikes the longer

edge of the rectangle, so that the sections are united by their longer sides rather than by the shorter. Crooked ribbons are caused by wedge-shaped sections, and are always to be avoided, because they make it difficult to economize space, and also because they present such a disorderly appearance. The knife, which should be placed at a right angle to the block and not obliquely, should strike the *whole edge* of the block at once, and should leave in the same manner.

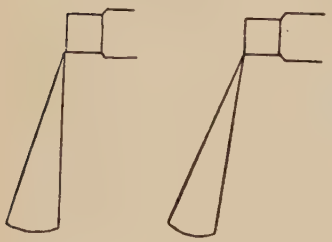

FIG. 18

If sections stick to the knife, it may be that the knife is too nearly parallel with the surface of the block, as in Fig. 18, *A*. By inclining the knife as in Fig. 18, *B*, this difficulty is often obviated. A split or scratch in the paraffin ribbon may be caused by a nick in the knife. Use some more favorable position of the edge, or sharpen the whole knife. A split or a scratch in the ribbon is often caused by some hard granule which becomes fastened to the inner side of the edge of the knife. This is the most common cause of the difficulty. Simply wipe the knife by an upward stroke of the finger. Do not use a cloth.

Sometimes good sections can be cut with a rather slow stroke when a rapid stroke fails. When paraffin is rather hard, sections may sometimes cut nicely at 5μ, when at 10μ the ribbons cannot be secured. If very thin sections are desired and the paraffin seems too soft, cool the paraffin and the edge of the knife with ice. Sometimes hard paraffin does not ribbon well. This difficulty may be remedied by dipping a hot needle in soft paraffin and applying it to the opposite edges of the block to be cut. Often the mere warming of the opposite edges of the block with a hot needle is sufficient.

FIXING SECTIONS TO THE SLIDE

Sections must be firmly fixed to the slide, or they will be washed off during the processes involved in staining. Mayer's albumen fixative is excellent for this purpose. Formula:

White of egg (active principle)	50 c. c.
Glycerine (to keep it from drying up) . . .	50 c. c.
Salycilate of soda (antiseptic, to keep out bacteria, etc.)	1 g.

Shake well and filter. It will kept from 2 to 6 months. The fixative may be used alone or in connection with the water method. Put a small drop of fixative on the slide, smear it evenly over the surface, and then wipe it off with a clean finger or piece of linen until only a scarcely perceptible film remains; then add several drops of distilled water and float the sections or ribbons on the water. Warm gently until the paraffin becomes smooth and free from wrinkles. Be careful not to melt the paraffin, for the albumen of the fixative coagulates with less heat than is required to melt the paraffin. It is a very good plan to put the slide on the top of the water-bath for a moment and then, after the sections have become smooth, remove the surplus water and leave them on the bath with a couple of thicknesses of blotting paper under them for 3 or 4 hours, or, better, over night. If the fixative is used alone, as is often the case when sections are very thick, none of this delay is necessary, since the sections are merely laid upon the fixative and pressed down gently with the finger.

REMOVAL OF THE PARAFFIN

To remove the paraffin it is very customary to heat the slide gently until the paraffin begins to melt, and then, holding the slide at an angle of about 45°, pour on a little turpentine or xylol. This should carry off the paraffin immediately. The reagent used in this first pouring cannot be used again. Now flood the slide several times with the turpentine or xylol, pouring the reagent back into the bottle. Overheating is ruinous. Many prefer not to heat the slide at all, but in this case a larger quantity of the reagent is required, and the process is unnecessarily slow. If the paraffin can come just to the melting-point without passing at all beyond it, no damage is done, and the removal of the paraffin is greatly facilitated. It is a good plan to warm the slide on the water-bath, or better still, on a bath like that shown in Fig. 7. Never attempt to warm the paraffin over a lamp.

REMOVAL OF XYLOL OR TURPENTINE

Remove the turpentine or xylol by flooding the slide with 95 per cent. alcohol. It will be remembered that in dehydrating, the absolute alcohol was poured off one or two times. This alcohol may be used for rinsing off the turpentine or xylol. One hundred c.c. of turpentine and 200 c.c. of 95 per cent. alcohol should be sufficient for fifty slides, even if the sections are to be mounted under the longest covers.

It was formerly a common practice to put the slide into a Stender dish of xylol to dissolve off the paraffin, and then with a dish of absolute alcohol to remove the xylol. Of course, the xylol was quickly charged with paraffin and the alcohol with xylol, so that the efficiency of both reagents was soon impaired. By keeping both reagents in bottles and pouring the liquid on the slide, the reagents are always fresh. A given quantity of the reagent will prepare as many slides by one method as by the other.

TRANSFER TO THE STAIN

After the paraffin has been removed with xylol or turpentine, and the xylol or turpentine has been rinsed off with alcohol, the slide may be placed directly into the stain. In case of aqueous stains, however, it is better to dip the slide into water in order to avoid carrying alcohol into the stain.

The method described in the previous edition, requiring the slide to be passed down through the alcohols until it reached an alcohol of about the same strength as the stain, has been found to be entirely unnecessary. The method did no damage, except that it entailed a great loss of time.

DEHYDRATING

After the sections have been stained, they must be dehydrated in 95 per cent. and absolute alcohol. A few seconds is usually sufficient for this step. The 95 per cent. alcohol is used here merely to save the more expensive absolute alcohol. The dehydration is best accomplished by dipping the slide into the alcohol. After forty or fifty preparations have been dehydrated, it is a good plan to fill another Stender dish with absolute alcohol; then

dip the slide into the 95 per cent. alcohol, then into the somewhat impaired absolute alcohol, and finally into the fresh absolute alcohol. In this way the absolute alcohol can be kept in efficient condition for a long time.

CLEARING

After the sections have been dehydrated, they must be cleared, or made transparent by some clearing agent. The clearing agent must be a solvent of balsam, but it is not at all necessary that the balsam shall be dissolved in the particular clearing agent which has been used. Xylol balsam is used not only when preparations have been cleared in xylol, but also when they have been cleared in clove oil, cedar oil, bergamot oil, or other clearing agents.

Clove oil is an excellent clearing agent. Take the slide from the absolute alcohol, wipe it on the back with filter paper and remove as much of the alcohol as possible, but never allow the preparation to become at all dry. Pour on a few drops of clove oil, and drain them off at once in such a way as to carry with them whatever alcohol may still remain. Then flood the slide repeatedly with clove oil, draining the clove oil back into the bottle. If judiciously used, 50 c.c. of clove oil is enough to clear one hundred preparations. Sections are usually cleared in a few seconds. The only objection to clove oil is that mounts harden slowly. To overcome this difficulty, the slide may be dipped in xylol before mounting in balsam, it may be placed upon the bath with a slide box cover or several thicknesses of pasteboard under it to protect it from too much heat. If the mount has been stained in gentian-violet, the clove oil should be followed by cedar oil or xylol before mounting in balsam.

Xylol is a very popular clearing agent. It is better to keep it in a Stender dish and dip the slide into it, or even let the slide stand in the dish for a few minutes. Xylol evaporates rapidly, and consequently there must be no delay in applying the balsam and the cover when the slide is removed from the xylol. Never allow the xylol to dry.

For clearing sections on the slide, other clearing agents are hardly worth mentioning.

MOUNTING IN BALSAM

After the sections are cleared, wipe the slide on the side which does not bear the sections. Put on a drop of Canada balsam and add a clean,[1] thin cover. Before the cover is put on, pass it through the flame of an alcohol lamp to remove moisture, for it would be a pity indeed to injure a preparation at this stage of the process. Add a label, and the mount is complete.

A TENTATIVE SCHEDULE FOR PARAFFIN SECTIONS

It will be useful to give several tentative schedules for the use of beginners. It cannot be too strenuously insisted that *these schedules are only tentative*, their sole object being to give the beginner a start. The following is a tentative schedule for the ovary of a lily at any period before fertilization. The pieces should not be more than half an inch in length.

1. Chromo-acetic acid, 1 day.
2. Wash in water, 1 day.
3. 15 per cent., 35 per cent., 50 per cent., 70 per cent., 85 per cent., 95 per cent. alcohol, 6 to 24 hours each, as convenient.
4. 100 per cent. alcohol, 24 hours. This should be changed once or twice. The volume should be at least five times that of the material.
5. Transfer from absolute alcohol to xylol, allowing at least 2 hours in each of the three mixtures, and 2 hours in pure xylol.
6. Add paraffin to the xylol and keep warm for 12 to 24 hours.
7. Melted paraffin in the bath, 2 to 24 hours, as convenient. The paraffin should be changed once or twice.
8. Imbed.
9. Section; about 10 μ is a good thickness.
10. Fasten to the slide.
11. Dissolve off the paraffin in turpentine or xylol.
12. Rinse off the turpentine or xylol with 95 per cent. alcohol.
13. Delafield's hæmatoxylin, 10 minutes.
14. Rinse in water, 5 minutes. A couple of hours does no harm.
15. Acid alcohol, 1 second.
16. 50 per cent. alcohol, 1 minute. An hour would do no harm.

[1] Slides and covers should be treated with hydrochloric acid, or equal parts of hydrochloric acid and water, for several hours. They should then be thoroughly rinsed in water and placed in 95 per cent. alcohol. They should be wiped with a cloth perfectly free from lint.

17. Erythrosin, 30 seconds to 1 minute.
18. Dehydrate in 95 per cent. and in absolute alcohol.
19. Clear in clove oil or xylol.
20. Mount in balsam.
21. Label.

That the paraffin method is tedious and complicated is universally recognized. Many substitutes have been tried, but with little success. Osterhout has recently revived and improved a method of imbedding in soap. He uses cocoanut oil soap. It is better to make one's own soap, using 70 c.c. of cocoanut oil to 38.5 c.c. of a 28 per cent. solution of caustic soda in water. The tissue, after the usual fixing and washing, is placed in warm water, and the soap is added gradually until a fairly strong solution is obtained. It may then stand in the bath for 2 or 3 days. When sufficiently firm, the block may be cut. The sections form a perfect ribbon, and do not crumble or crush like paraffin ribbons. They may be fixed to the slide with albumen. When firmly fixed to the slide, the soap is dissolved off with water. The preparation is then stained in the usual way. Osterhout's account is published in the *University of California Publications, Botany.* **2** : 73–90. 1904.

CHAPTER X

THE CELLOIDIN METHOD

The celloidin method is used more extensively by zoölogists than by botanists. Where many mounts are necessary and only a single section is needed for each mount, the method is to be recommended, if the sections cannot be cut equally well without any imbedding. All the sections can be stained and cleared at one time, so that, in making the individual mounts, it is necessary only to place a section on the slide and add a drop of balsam and a cover. Another advantage, and the only one so far as the botanist is concerned, is that hard roots and stems, which cannot be handled by the paraffin method, are cut easily in celloidin. Where serial sections are necessary, as in most morphological and cytological work, the method is too tedious to be worth even a trial, unless the sections cannot be cut in any other way. Besides, most of the more valuable stains color the celloidin matrix, and if the matrix be removed, the more delicate elements may be displaced or even lost.

Celloidin and collodion are forms of nitro cellulose. They are inflammable, but do not explode. Schering's celloidin, which is only a collodion prepared by a patented process, is in general use for imbedding. Granulated and shredded forms of celloidin are on the market, but the tablets are more convenient. Directions for making the various solutions accompany the celloidin. To make a 2 per cent. solution, add to one tablet enough ether alcohol to make the whole weigh 2,000 g. To make a 4 per cent. solution, add another tablet, and to make a 6 per cent. solution, add an additional tablet, and so on.

The collodion method was published by Duval[1] in 1879. Celloidin was recommended by Merkel and Schiefferdecker[2] in

[1] Duval, *Journal de l'anatomie*, 1879, p. 185.

[2] Merkel and Schiefferdecker, *Archiv für Anatomie und Physiologie*, 1882.

1882. The principal features of the method are as follows: Material is dehydrated in absolute alcohol; treated with ether-alcohol; infiltrated with celloidin; imbedded in celloidin; hardened in chloroform or alcohol; after which it is cut, stained, and mounted.

Eycleshymer, who brought the celloidin method to a high degree of efficiency, published in 1892 a short account, which may be summarized as follows: Put the celloidin tablet, or fragments, into a wide-mouthed bottle, and pour on enough ether-alcohol (equal parts ether and absolute alcohol) to cover the celloidin. Occasionally shake and add a little more ether-alcohol until the celloidin is all dissolved. The process may require several days. The solution should have the consistency of a very thick oil. Label this solution No. 4. Solution No. 3 is made by mixing two parts of solution No. 4 with one part of ether-alcohol. Solution No. 2 is made by mixing two parts of No. 3 with one part of ether-alcohol. Solution No. 1 consists of equal parts of ether and absolute alcohol.

After dehydrating, the material is placed successively in solutions 1, 2, 3, and 4. For an object 2 mm. square, 24 hours in each solution is sufficient; for the brain of a cat, a week is not too long.

A paper tray may be used for imbedding. Pour the object, with the thick solution, into the tray and harden in chloroform for 24 hours; then cut away the paper and place the block in 70 per cent. alcohol for a few hours. The material may be left indefinitely in a mixture of equal parts of 95 per cent. alcohol and glycerine.

Before cutting, the object is mounted upon a block of wood. A block, suited to the microtome clamp, is dipped in ether-alcohol, which removes the air and insures a firmer mounting. Dip the block of wood in solution No. 3, and the piece of celloidin containing the object in solution No. 1. Press the two firmly together, and place in chloroform until the joint becomes hardened.

Set the blade of the microtome knife as obliquely as possible. Both the object and the knife should be kept flooded with 70 per cent. alcohol, and the sections, as they are cut, should be transferred to 70 per cent. alcohol.

Stain in Delafield's hæmatoxylin for 5–30 minutes. Wash in water for about 5 minutes, and then decolorize in acid alcohol (2–5 drops of hydrochloric acid to 100 c.c. of 70 per cent. alcohol) until the stain is extracted from the celloidin, or at least until the celloidin retains only a faint pinkish color. Wash in 70 per cent. alcohol (not acid) until the characteristic purple color of the hæmatoxylin replaces the red due to the acid. Stain in eosin (preferably a 1 per cent. solution in 70 per cent. alcohol) for 2–5 minutes. Dehydrate in 95 per cent. alcohol for about 5 minutes. Absolute alcohol must not be used, unless it is desirable to remove the celloidin matrix. Eycleshymer's clearing fluid (equal parts of cedar oil, bergamot oil, and carbolic acid) clears readily from 95 per cent. alcohol. Mount in balsam.

If serial sections are necessary, arrange the sections upon a slide, using enough 70 per cent. alcohol to keep the sections moist, but not enough to allow them to float. Cover the sections with a strip of toilet paper, which can be kept in place by winding with fine thread. After the sections have been stained and cleared, remove the excess of clearing fluid by pressing rather firmly with a piece of blotting-paper. Then remove the toilet paper and mount in balsam.

With occasional slight modifications, we have used the method as presented by Eycleshymer in his classes. Instead of the graded series of celloidin solutions, we use a 2 per cent. solution, which is allowed to concentrate slowly by removing the cork occasionally, or by using a cork which does not fit very tightly. The material is imbedded when the solution reaches the consistency of a very thick oil. If the material is to be cut immediately, we prefer to imbed it and fasten it to the block at the same time. The blocks should have surface enough to accommodate the objects, and should be about one-fourth of an inch thick. White pine makes good blocks; cork is much inferior. Place the block for a moment in ether-alcohol and then dip into the 2 per cent. celloidin the end of the block which was left rough by the saw. With the forceps remove a piece of the material from the thick celloidin and place it upon the block, taking care to keep it right side up. Dip the

block with its object first in thick celloidin, then in thin, and after exposing to the air for a few minutes drop it into chloroform, where it should remain for about 10–20 hours. It should then be placed in equal parts of glycerine and 95 per cent. alcohol, where it may be kept indefinitely. If the material is hard, like many woody stems, it will cut better after remaining in this mixture for a couple of weeks.

The following schedules, beginning with the celloidin sections in 70 per cent. alcohol, will give the student a start in the staining:

Delafield's Hæmatoxylin and Eosin.—

1. 70 per cent. alcohol, 2–5 minutes.
2. Delafield's hæmatoxylin, 5–30 minutes.
3. Wash in water, 5 minutes.
4. Acid alcohol (1 c.c. hydrochloric acid + 100 c.c. of 70 per cent. alcohol) until the stain is extracted from the celloidin, or at least until only a faint pinkish color remains.
5. Wash in 70 per cent. alcohol (not acid) until the purple color returns.
6. Stain in eosin (preferably a 1 per cent. solution in 70 per cent. alcohol), 2–5 minutes.
7. Dehydrate in 95 per cent. alcohol, 2–5 minutes. Do not use absolute alcohol unless you wish to dissolve the celloidin, which is not necessary with this staining.
8. Clear in Eycleshymer's clearing fluid, usually 1–2 minutes, but sometimes 5–10 minutes.
9. Mount in balsam.

Safranin and Delafield's Hæmatoxylin.—

1. 70 per cent. alcohol, 2–5 minutes.
2. Safranin (alcoholic), 6–24 hours.
3. Acid alcohol (a few drops of hydrochloric acid in 70 per cent. alcohol) until the safranin is removed from the cellulose walls.
4. Wash in 50 per cent. alcohol, 5–10 minutes to remove the acid.
5. Delafield's hæmatoxylin, 2–5 minutes.
6. Wash in water, 5 minutes.
7. Acid alcohol, a few seconds.
8. Dehydrate in 95 per cent. alcohol, 2–5 minutes, then in absolute alcohol, 2–5 minutes, which will partially dissolve the celloidin.
9. Clear in clove oil, which will complete the removal of the celloidin.
10. Be sure that the sections are free from fragments of celloidin and then mount in balsam.

Jeffrey's improvements in the celloidin method have been described in considerable detail by Plowman.[1] Sections of hard stems and roots cut by this method could hardly be surpassed, and they are perfectly adapted to the requirements of photomicrography. The following is a brief abstract of Plowman's paper:

1. **Preparation of Material.**—Dead and dry material should be repeatedly boiled in water and cooled to remove air. An air-pump may be used in addition. Living material may be fixed in a mixture of picric acid, mercuric chloride, and alcohol:

Mercuric chloride, saturated solution, in 30 per cent. alcohol . 3 parts
Picric acid, saturated solution, in 30 per cent. alcohol . . 1 part

Fix 24 hours, and wash by passing through 40, 50, 60, 70, and 80 per cent. alcohol, allowing each to act for 24 hours.

2. **Desilification, etc.**—Silica and other mineral deposits are removed by treating with a 10 per cent. aqueous solution of commercial hydrofluoric acid. The material is transferred to this solution from water or from the 80 per cent. alcohol. The process may require 3 or 4 days, with one or two changes of the acid and frequent shaking of the bottle. An ordinary wide-mouthed bottle, coated internally with hard paraffin, should be prepared, since the acid is usually sold in bottles with narrow necks. The bottles are easily prepared by filling them with hot paraffin and simply pouring the paraffin out. Enough will stick to the bottle to protect the glass from the acid. Wash in running water 3 or 4 hours.

3. **Dehydration.**—Use 30, 50, 70, 90, and 100 per cent. alcohol, allowing 12 hours in each grade.

4. **Infiltration with Celloidin.**—There should be ten grades of celloidin: 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 per cent. Transfer from absolute alcohol to the 2 per cent. celloidin. (We should prefer a previous treatment with ether-alcohol.) The bottle should be nearly filled, and the stopper should be clamped or wired in place. Put the bottle on its side in a paraffin bath at 50° to 60° C. for 12–18 hours. Cool the bottle quickly in cold water, taking

[1] Plowman, A. B., The Celloidin Method with Hard Tissues, *Botanical Gazette*, **37**: 456–461, 1904.

care that the water does not get into the bottle. Pour out the 2 per cent. solution (which, as well as all the other solutions, may be used repeatedly), and replace it with the 4 per cent., and proceed in the same way with the other grades. When the 20 per cent. solution is reached, a further thickening is reached by adding a few chips of dry celloidin from time to time until the mixture is quite stiff and firm. Remove each block with the celloidin adhering to it and harden it in chloroform for 12 hours. Then transfer to a mixture of equal parts of glycerine and 95 per cent. alcohol, where the material should remain for a few days before cutting.

5. **Cutting, Staining, and Mounting.**—Although 10 μ is usually thin enough, sections are readily cut as thin as 5 μ by this method. Remove the celloidin before staining by treating 10–15 minutes with ether; then wash in 95 per cent. alcohol and transfer to water, and then to the stain. Stain to a fairly dense purple in an aqueous solution of Erlich's hæmatoxylin; wash in dilute aqueous solution of calcium or sodium carbonate, and then in two changes of distilled water. Add a few drops of alcoholic solution of equal parts of Grübler's alcoholic and aqueous safranin, and stain to a rich red. A dilute stain acting 1–2 hours is better than a more concentrated stain acting for a shorter time. Transfer directly to absolute alcohol, clear in xylol, and mount in balsam.

Haidenhain's iron-hæmatoxylin is a very satisfactory stain for photographic purposes.

The celloidin method has its disadvantages as well as its advantages. It is extremely slow and tedious, and it is rarely possible to cut sections thinner than 10 μ, while, on the other hand, it gives smoother sections.

Succulent tissues, which are usually damaged by the paraffin method, are easily handled without any injury in celloidin. The fact that the method may be used without heat is often a further advantage. Stems and roots which cannot be handled at all in paraffin cut well in celloidin, and much larger sections can be cut than in paraffin.

When material is to be imbedded, use celloidin as a last resort. Use paraffin when you *can*, celloidin when you *must*.

CHAPTER XI

SPECIAL METHODS

While the methods already described are sufficient for most of the routine work of the ordinary laboratory, special methods are often necessary for special cases. In nearly every piece of research work the investigator will find some modification necessary before he can secure the best results. A few methods designed to meet special difficulties are given in this chapter.

VERY LARGE SECTIONS

It is sometimes desirable to cut very large sections. Sections as large as a corn stalk may be cut free hand or in celloidin. A section of a stem of *Zamia* two inches in diameter is difficult to handle by the usual methods. If a large microtome, such as is used in cutting complete sections of large brains, is available, the piece of stem is easily held for the cutting. We have cut such sections on a small sliding microtome. With a small microtome, which, of course, does not have a clamp large enough to hold such an object, one person must hold the stem firmly in place with his hands while another person cuts the section. With a rather soft stem, like *Zamia*, the surface must be flooded with 95 per cent. alcohol after each section. This hardens the tissues so that sections can be cut as thin as 15μ. About 20–30μ, however, is as thin as one ought to expect. Such sections properly stained and mounted between lantern slide covers, are very instructive. They may be used directly as lantern slides. Sections of the stem of the tree fern, *Alsophila*, have been cut as thin as 15μ in this way. Sections of these large ferns make good mounts without any staining. Flooding with strong alcohol facilitates the cutting of corn stems, since the rigid bundles are likely to tear the soft ground tissue, unless this has been hardened. A slight hardening is sufficient, so that about four or five sections can be cut in a minute.

STONY TISSUES

Sections of the stony tissues of hickory nuts, walnuts, peach stones, and similar refractory substances cannot be cut by ordinary methods.

With a fine saw, saw sections about 1 mm. in thickness. Rub a section between two pieces of fine sandpaper until it is not more than half a millimeter in thickness. Then rub it between two smooth hones, keeping the hones wet with water. When the section is thin enough, wash it thoroughly in water, using a pipette to rinse off any particles of dirt. Dehydrate in absolute alcohol, clear in clove oil, and mount in balsam. The long, narrow pores show better without any clearing. In this case, dry the section thoroughly, heat a few drops of balsam on the slide to drive off the solvent, put the section into the balsam, and add a cover. The air caught in the long, narrow pores will make them appear as black lines. Sections of most nuts show excellent detail without any staining. Thin sections, however, may be stained in the usual way.

Where a lathe is available, a section cut with the saw may be cemented to a piece of thick glass or any convenient surface with shellac. Grind one side smooth and polish it; then remove the section by dissolving the shellac with alcohol, and again fasten the section to the glass with shellac, and grind and polish the other side. Free the section by dissolving the shellac, and proceed as before.

After one side has been ground and polished, the section may be fastened to a slide by as thin a layer of shellac as possible. When the second side of the section has been ground, polished, and cleaned, a drop of absolute alcohol, a drop of shellac, and a cover may be added, and the mount is complete.

The sectioning of fossil plants has a technique of its own. The apparatus required is complicated and expensive. In general, it may be said that thin sections are sawed, and then ground and polished on a revolving disk. Such sectioning is reaching a high degree of perfection, sections of silicified wood showing almost as much detail of structure as sections of living wood. From

silicious fossils sections have been cut two inches square, and so thin as to admit of satisfactory examination under high powers. Directions could be given only by an expert in this line of work. Some investigators employ professional lapidaries.

THICK SECTIONS

It is sometimes desirable to make very thick sections to show general topography rather than detail. A longitudinal section of the fully grown ovule of *Ginkgo* or a cycad may be cut as thick as 3–5 mm. so as to include the entire group of archegonia. A slab can be cut from each side of the ovule with a fine saw, and a razor can be used for smoothing. If the section is from fresh material, it should be fixed, washed etc., with about the same periods as if it were to be imbedded in paraffin. When thoroughly cleared in xylol, the section should be put into a flat museum jar of suitable size and kept in xylol. No staining is desirable.

SCHULTZE'S MACERATION METHOD

Various solutions are used to separate a tissue into its individual cells. These solutions dissolve or weaken the middle lamella so that the cells are easily shaken or teased apart. Schultze used strong nitric acid and potassium chlorate. Put the material, which should be in very small pieces, into a test-tube; pour on just enough nitric acid to cover it, and then add a few crystals of potassium chlorate. Heat gently until bubbles are evolved, and let the reagent act until the material becomes white. Four or 5 minutes should be sufficient. The fumes are disagreeable and are very injurious to microscopes. Pour the contents of the tube into a dish of water. After the material is thoroughly washed in water, it may be teased with needles and mounted, or it may be put into a bottle of water and shaken until the many of the cells become dissociated.

After a thorough washing in water, the material may be stained. The large tracheids of ferns, dissociated in this way and stained in safranin or methyl green, make beautiful preparations.

PROTOPLASMIC CONNECTIONS

In exceptional cases, like the sieve plates of the Cucurbitaceae, the protoplasmic connections show plainly with ordinary methods, but in most cases it is necessary to resort to special methods in order to demonstrate protoplasmic continuity. In these special methods a reagent is used which causes the membranes to swell before the stain is applied. It is only by such an exaggeration that the more delicate connections can be shown.

Put thin sections of fresh material into a mixture of equal parts of sulphuric acid and water; and allow the reagent to act for 2–10 seconds. Wash the acid out thoroughly in water and stain in anilin blue. According to Gardiner, this stain should be made by adding 1 g. of the dry stain to 100 c.c. of a saturated solution of picric acid in 50 per cent. alcohol. The staining solution is then washed out in water, and the sections are mounted in glycerine. The sections may be dehydrated, cleared in clove oil, and mounted in balsam. The anilin blue may be used in 50 per cent. alcohol acidulated with a few drops of acetic acid.

Chloroïodide of zinc may be used instead of sulphuric acid. Treat the fresh sections for 2 hours with the iodine and potassium-iodide solution used in testing for starch; then treat about 12 hours with chloroïodide of zinc. Wash in water and stain in anilin blue. Examine in glycerine.

Meyer's pyoktanin method is one of the best. The reagents are as follows:

1. Iodine, potassium iodide solution: iodine 1 part, potassium iodide 1 part, water 200 parts.
2. Sulphuric acid 1 part, and water 3 parts; this mixture to be saturated with iodine.
3. Pyoktanin cœruleum 1 g., water 30 c.c. This pyoktanin is a very pure methyl violet obtained trom E. Merck in Darmstadt.

Put sections of the date seed into a watch-glass full of the first solution, and allow it to act for a few minutes; then mount in a drop of the solution. The connections will be only very

faintly stained, showing a slightly yellowish color. At the edge of the cover, add a drop of the second solution. The preparation will darken a little. Then allow a small drop of the third solution to run under the cover. Allow the stain to act for about 3 minutes. Then plunge the whole preparation into water. The action should be stopped before the entire section has become blue. Now wash the section quickly. If there are annoying, granular precipitates, remove them with a soft brush. Mount in glycerine. The membrane should be a clear blue, while the protoplast and connections should be a blue-black.

The following is Strasburger's modification of Meyer's method, and shows the connections with great distinctness:

1. Treat the fresh sections with 1 per cent. osmic acid 5–7 minutes.
2. Wash in water 5–10 minutes.
3. Treat with a solution of iodine in potassium iodide (0.2 per cent. iodine and 1.64 per cent. potassium iodide), 20–30 minutes.
4. Transfer to 25 per cent. sulphuric acid, which should act for at least half an hour; 24 hours may be necessary.
5. Bring the sections into 25 per cent. sulphuric acid which has been saturated with iodine. Add a drop of Meyer's pyoktanin solution (1 g. pyoktanin coeruleum as sold by E. Merck in Darmstadt in 30 c.c. of water).

In about 5 minutes the sections will be stained sufficiently and can be examined in glycerine. If there are annoying precipitates, remove them with a soft brush.

According to Meyer, the swelling is an advantage only when the walls are very thin. When the walls are thick, the connections show better without any previous swelling.

Try the following method with the seeds of *Latania*, *Chamerops*, *Phoenix*, or *Phytelephas*: Soak in water and cut thin sections. Extract the oily and fatty substances with xylol; wash in 95 per cent., or in absolute alcohol; stain in anilin blue (Hoffman's blue 1 g. dissolved in 150 c.c. of 50 per cent. alcohol) for

a few minutes. Examine in glycerine. This method succeeds very well with seeds of the date, which is sold at all groceries.

Permanent preparations may be secured by the following method:

1. Fix in 1 per cent. osmic acid, or in absolute alcohol, 5–10 minutes.
2. Stain for 24 hours in Delafield's hæmatoxylin.
3. Wash for a few minutes in acid alcohol (5 drops of hydrochloric acid in 50 c.c. of 70 per cent. alcohol).
4. Wash for a few minutes in ammonia alcohol (5 drops of ammonia to 50 c.c. of 70 per cent. alcohol).
5. Dehydrate in absolute alcohol, clear in xylol, and mount in balsam.

STAINING CILIA

The cilia of the large spermatogoid of *Ginkgo* and the cycads take a brilliant stain with gentian-violet, whether the gentian-violet be used alone or in combination with safranin. The cilia of the spermatozoids of the pteridophytes also stain by this method, although not so brilliantly as in case of the cycads.

The cilia of the motile spores of Thallophytes may often be demonstrated by allowing a drop of the iodine solution used in testing for starch to run under the cover.

Zimmermann gives the following method: Bring the objects into a drop of water on the slide and invert the drop over the fumes of 1 per cent. osmic acid for 5 minutes. Allow the drop to dry. Then add a drop of 20 per cent. aqueous solution of tannin, and after 5 minutes wash it off with water. Stain in a strong aqueous solution of fuchsin (or carbol fuchsin) for 5 minutes. Allow the preparation to dry completely, and then add a drop of balsam and a cover. The cilia should take a bright red.

Zimmermann also found the following method satisfactory for the cilia of the zoöspores of algæ and fungi: Fix by adding a few drops of 1 per cent. osmic acid to the water containing the zoöspores; then add an equal amount of a mixture of fuchsin and

methyl violet. The fuchsin and methyl violet should be 1 per cent. solutions in 95 per cent. alcohol. In a few seconds the cilia stain a bright red.

VASCULAR BUNDLES IN LIVING TISSUES

In studying venation, and in tracing the course of vascular bundles in large ovules and in other organs, it is often an advantage to use a stain. If a stem of *Impatiens* be cut under water, and the cut surface be then placed in a dilute aqueous solution of eosin, the eosin will rise in the vessels, making them very prominent. The outer bundles of the large ovules of cycads are very easily studied by this method. The inner bundles also may be seen by opening the seed and removing the endosperm.

If such preparations could only be cleared, they would be still more valuable, but the effect is due to the presence of the staining fluid in the vessels, and any subsequent treatment diffuses or destroys the stain. Perhaps a little experimenting might obviate the difficulty.

STAINING LIVING STRUCTURES

Some stains will stain living structures. Cyanin, methyl blue, and Bismarck brown have been recommended for this purpose. The solutions should be very dilute, not stronger than 1:10,000 or 1:500,000. The solutions should be very slightly alkaline, never acid. It is claimed that such solutions never stain the nucleus, and that if the nucleus stains at all, it is an indication that death is taking place.

Campbell succeeded in staining the living nuclei in the stamen hairs of *Tradescantia* by using dilute solutions of dahlia and of methyl violet (0.001 to 0.002 per cent. in water). Dividing nuclei were stained.

For determining the stage of development of fresh material it is often necessary to use a stain. For this purpose stronger stains may be used, since it is unimportant whether the tissue is killed or not. An aqueous solution of methyl green or eosin can be recommended. With 1 per cent. solutions, diluted one-half with water, mitotic figures can be recognized with ease.

PART II

In the preceding chapters the principles and methods of technique have been described in a general way. It is difficult, especially for a beginner, to apply general principles to specific cases, and, besides, the types which he might select for the preparations might not form a symmetrical collection. Consequently, a series of forms has been selected which will not merely serve for practice in microscopical technique, but will also furnish the student with preparations for a fairly satisfactory study of plant structures from the algæ up to the angiosperms. It is not at all our purpose to discuss general morphology, but rather to answer, by means of sketches and specific directions, the multitudinous questions which confront the instructor in the laboratory. For those who have had a thorough training in general morphology the following suggestions will be in some degree superfluous. Those who are beginning the study of minute plant structure are referred to the standard textbooks for descriptions of the plants mentioned here.

The directions for collecting and growing laboratory material constitute an important feature of this part of the book.

With a few exceptions, the order in which the forms are presented is that given in Engler's *Syllabus der Pflanzenfamilien.*

CHAPTER XII

MYXOMYCETES AND SCHIZOPHYTES

MYXOMYCETES

With the exception of a few forms like *Fuligo* (often found on oak stumps and on oak bark in tan yards), the myxomycetes are small, and are usually overlooked by collectors (Fig. 19). A careful examination of rotting logs in moist woods will usually reveal an abundance of these delicate and beautiful organisms. Various species may be found in spring, summer, and autumn. The plasmodia are most abundant just after a warm shower. A couple of days of dry weather will then bring sporangia in abundance. The specimens should be pinned to the bottom of the box for safe carrying. An excellent collecting-box can be made from an ordinary paper shoe-box. On the bottom of the box place a thin piece of soft pine, or a piece of the corrugated paper so commonly used in packing; or, better still, a sheet of cork. At each end nail in a piece of pine half an inch thick and an inch high. Upon these end pieces place a thin piece of pine, thus making a second bottom, which, of course, should not be fastened. A second pair of ends with a third pine bottom nailed to them may rest upon the second bottom. The three bottoms will give a considerable surface upon which the material may be pinned. For most purposes, the specimens are simply allowed to dry, and are then fastened with glue or paste to the bottom of a small box.

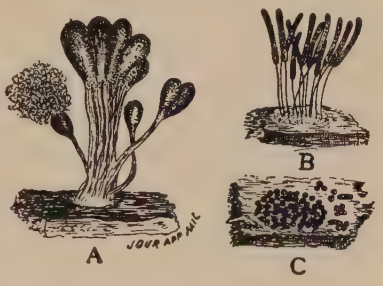

FIG. 19.—Myxomycetes

Growing on rotten wood. *A*, *Hemitrichia rubiformis*. ×20. *B*, *Stemonitis ferruginea*. Natural size. *C*, *Trichia varia*. ×1½.

Plasmodia and young sporangia may be fixed in chromo-acetic acid or Flemming's fluid. Sections are easily cut in paraffin, and

should not be more than 5 μ in thickness, and should be thinner, if possible. The safranin, gentian-violet, orange combination, or the iron-hæmatoxylin may be used for staining.

Spores of most myxomycetes can be germinated in water in a hanging-drop culture, as described on page 58. Plasmodia may be raised by sowing spores on moist, rotten bark or wood and placing the culture under a bell jar, where the moist, sultry condition favorable to the growth of plasmodia is easily imitated. Plasmodia may be got upon the slide by inclining the slide at an angle of about 15°, with one end of the slide at the edge of a plasmodium, and allowing water to flow gently down from the upper end of the slide to the lower. The proper flow of water could be secured by dropping water from a pipette, but a less tedious plan is to arrange a siphon so as to secure a similar current. The plasmodium will creep up the slide against the current, furnishing an excellent illustration of rheotropism. Enough plasmodium for an examination may be formed in 2 or 3 hours. Examined under the microscope, the preparation should give an excellent view of the streaming movements of protoplasm.

The following is another method for getting the plasmodia upon the slide: Place the slides on a pane of glass, and upon each slide place a small piece of plasmodia-bearing wood. Cover with a bell jar. Wet blotting-paper or a small dish of water included under the jar will help to create the warm, sultry atmosphere. The slides may be covered with plasmodium in a few hours. Permanent preparations may be made by immersing the slide in chromo-acetic acid, then washing and staining without removing the plasmodium from the slide. Acid fuchsin is a good stain for bringing out the delicate strands of the plasmodium. Iron-hæmatoxylin followed by acid fuchsin or erythrosin brings out both nuclei and cytoplasmic strands.

Some of the foregoing methods are taken from an article by Prof. Howard Ayers in the January and February (1898) numbers of the *Journal of Applied Microscopy*. Other methods, with directions for various experiments, are given in the same article.

SCHIZOPHYTES (*Fission Plants*)

BACTERIA (*Schizomycetes, Fission Fungi*)

The methods of modern bacteriological technique are so numerous and so specialized that we must refer to laboratory manuals for instruction in this subject. The method given here will merely enable the student to study the form and size of those bacteria which are more easily demonstrated.

Foul water at the outlets of sewers and such places will usually afford an abundance of *Coccus*, *Bacillus*, *Spirillum*, and *Beggiatoa* forms. Place a drop of water on a slide, heat it gently until the water evaporates, then stain with fuchsin or methyl violet, dehydrate, clear in xylol, and mount in balsam (Fig. 20).

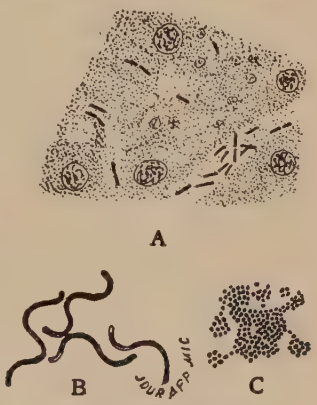

FIG. 20.—Bacteria. ×535

A, *Bacillus anthracis*, from a paraffin section cut from the liver of a mouse. Fixed in chromo-acetic acid, stained in methyl violet and Bismarck brown, and mounted in balsam. *B*, *Spirillum* sp. From a preparation stained in fuchsin. *C*, *Staphylococcus pyogenes* aureus. From a preparation stained in gentian-violet.

The hay infusion is a time-honored method for securing bacteria for study. Pour hot water on a handful of hay, and filter the fluid through blotting-paper. Place the fluid in a glass dish, and cover with a piece of glass to keep out the dust. When the fluid begins to appear turbid, bacteria will be abundant. The active movements are easily observed in a mount from the turbid water. As the bacteria pass into the resting condition, they form a scum on the surface of the water. Usually the first to appear is a somewhat rod-shaped form, the *Bacterium termo* of the older texts. *Spirillum* and *Coccus* forms often appear later.

Fine preparations may be obtained by inoculating a mouse with *Anthrax* or some other form, and then cutting paraffin sections of favorable organs. For making mounts of a dangerous form like *Anthrax*, it is better to secure properly fixed material from a bacteriologist. Gentian-violet with a faint Bismarck brown for a background makes a good combination. The following schedule gives good results with *Anthrax* and many other bacteria:

1. Gentian-violet, 5 minutes.
2. Rinse in water a few seconds.
3. Gram's solution (iodine 1 g., potassium iodide 2 g., water 300 c.c.) until the color is almost or quite black; this will generally require 1 or 2 minutes.
4. 95 per cent. alcohol until the color has nearly disappeared.
5. Rinse in water and examine. If the bacteria are well stained, a counter-stain for the background may be added.
6. Erythrosin, 3 or 4 seconds; or Bismarck brown 5 or 10 seconds.
7. 95 and 100 per cent. alcohol, dehydrating as rapidly as possible. Not more than 5 or 10 seconds can usually be allowed.
8. Xylol.
9. Balsam.

Leptothrix may often be obtained by scraping the inside of the cheek. *Beggiatoa*, a form with oscillating movements like *Oscillatoria*, is often found in foul water. Its presence may be indicated by whitish patches on the bottom.

A few years ago Fischer claimed that the Bacteria and Cyanophyceae do not possess even the morphological forerunner of a nucleus. It is now quite generally believed that the large "central body" of the Cyanophyceae, a structure which can be seen with a dry lens, is a nucleus, which differs from the nucleus of higher plants chiefly in having no nuclear membrane or nucleolus. Whether the Bacteria have a nucleus or not is still to be demonstrated. The student need not be disappointed if even the larger Bacteria fail to show a nucleus.

CYANOPHYCEAE. BLUE-GREEN ALGÆ. (*Schizophyceae, Fission Algæ.*)

The blue-green algæ include unicellular, colonial and filamentous forms. They occur everywhere in damp or wet places. On the vertical faces of rocks where there is a constant dripping of water, brilliant blue-green forms are abundant. In the Yellowstone National Park the brilliant coloring of the rocks is due in large measure to members of this group. Many forms occur as brownish or greenish gelatinous layers on damp ground or upon rocks, or even upon damp wooden structures in greenhouses. Other forms float freely in water.

Oscillatoria.—For most purposes it is best to study *Oscillatoria* in the living condition. It is readily found in watering troughs, in stagnant water, on damp earth, and in other habitats. The commonest forms have a deep blue-green or brownish color. It is very easy to keep *Oscillatoria* all the year in the laboratory. Simply put a little of a desirable form into a gallon glass jar half filled with water. By adding water occasionally to compensate for evaporation, the culture should keep indefinitely. In a jar with a tightly fitting cover we have kept such a culture for years without renewing the water.

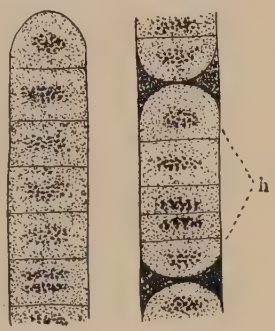

FIG. 21.—*Oscillatoria*

Portions of two filaments, the one at the right showing an hormogonium, *h*. Magdala red and anilin blue, mounted whole in Venetian turpentine.

For the purposes of identification and herbarium specimens, the material may simply be placed on a slip of mica and allowed to dry. When wanted for use, add a drop of water and a cover, and the mount is ready for examination. For sections or for mounts in Venetian turpentine or glycerine, fix the material in chromo-acetic acid (Fig. 21).

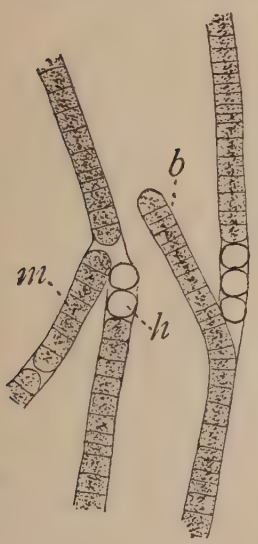

FIG. 22.—*Tolypothrix*
b, a false branch. *h*, heterocysts.

Tolypothrix.—This form occurs as small tufts, either floating in stagnant water or attached to plants and stones. Some species grow upon damp ground. It furnishes an excellent example of false branching (Fig. 22). *Scytonema* is a similar form which is fairly common. The Venetian turpentine method or the glycerine method should be employed for permanent preparations, but this, like all small filamentous algæ, may be dried on mica for herbarium purposes.

Nostoc.—*Nostoc* is a cosmopolitan form. It occurs on damp earth or floating freely in water. Young specimens are generally in the form of gelatinous nodules but in older specimens the

form may be quite various. It is very easy to make sections, since the gelatinous matrix cuts well and holds the filaments together. Chromo-acetic acid is a good fixing agent. Stains which stain the gelatinous matrix make the preparations look untidy, but may give the best views of the cell contents.

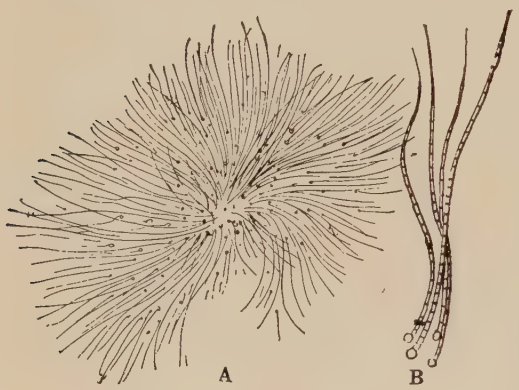

FIG. 23.—*Rivularia*

A, nodule crushed under cover-glass. *B*, four filaments more highly magnified, showing heterocysts at the base.

Small nodules may be stained in bulk and got into Venetian turpentine. Crushed under the cover, they make instructive preparations.

Rivularia.—This form is readily found on the underside of the leaves of water-lilies (*Nuphar*, *Nymphaea*, etc.), but is also abundant on submerged leaves and stems of other plants. It occurs in the form of translucent, gelatinous nodules of various sizes. Chromo-acetic acid gives beautiful preparations, but good results can also be secured from formalin or picric-acid material.

The most instructive preparations for morphological study can be obtained by the glycerine method. Stain in eosin or Mayer's hæm-alum. When ready for mounting, crush a small nodule by pressing on the cover-glass. Fig. 23 is drawn from such a preparation. The paraffin method is easily applied, since the gelatinous matrix keeps the plants in place. Any form of similar habit may be prepared in the same way.

Gloeotrichia.—*Gloeotrichia* (Fig. 24) is a free-floating form. The nodules, when young, are firm like *Nostoc*, but as they grow older and larger, they become hollow and soft. The older forms become so much dissociated that they lose their characteristic form and merely make the fixing fluid look turbid. Allow a drop

of such material to spread out and dry upon a slide which has been slightly smeared with albumen fixative. Dip a few times in 95 per cent. alcohol, stain in safranin, gentian-violet, orange, or in cyanin and erythrosin, and mount in balsam. Elegant preparations may be made in this way.

The firmer nodules may be treated like *Nostoc* or *Rivularia*.

Fig. 24.—*Gloeotrichia*

Photomicrograph from a preparation stained in cyanin and erythrosin. Negative by Dr. W. J. G. Land.

Wasserblüthe.—Many genera of the Cyanophyceae occur as scums, often iridescent, on the surface of stagnant or quiet water. Some of the commonest forms are *Coelosphaerium* and *Anabaena* (Fig. 25). Some of the Chlorophyceae also occur as *Wasserblüthe*. Where the material is very abundant, it may be collected by simply skimming it off with a wide-mouthed bottle, but where it is rather scarce, it is better to filter the water through a cloth, and finally rinse the algæ off into a bottle. Enough formalin may now be added to the water in the bottle to make a 3 per cent. solution. The material may be kept here indefinitely, but after a few hours it is ready for use. If the forms are small, like *Anabaena*, smear a slide lightly with Mayer's albumen fixative, as if for paraffin sections, add a drop of the material and allow it to dry, heat the slide gently to coagulate the albumen, or immerse the slide in strong alcohol for a few minutes, and then proceed

with the staining. Cyanin and erythrosin is a good combination for differentiating the granules. Delafield's hæmatoxylin, used alone, stains some granules purple and others red. Iron alum-hæmatoxylin is excellent for heterocysts. If the forms are large enough to collapse with such treatment the Venetian turpentine method or the glycerine method may be employed.

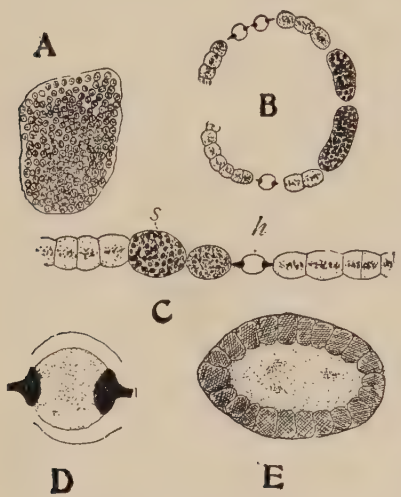

FIG. 25.—Wasserblüthe

A, *Coelosphaerium Kuetzingianum*. *B*, *Anabaena flos-aquae*. *C*, *Anabaena gigantea*. *D* and *E*, a heterocyst and spore of *A. gigantea* drawn from paraffin sections stained in cyanin and erythrosin.

If it is desirable to make paraffin sections, put the material, drop by drop, on a piece of blotting-paper until an appreciable layer of sediment is obtained. Get the paper with its material into paraffin by the usual method, taking great care not to wash the algæ off. After imbedding, trim away the paper and dip the block in melted paraffin. Sections can now be cut and stained in the usual manner.

CHAPTER XIII

CHLOROPHYCEAE. GREEN ALGÆ

The green algæ are found in both fresh and salt water, but are most abundant in fresh water. The ponds, ditches, and rivers of any locality will yield an abundance and variety both of the unicellular and the multicellular members of this group. Most of the forms are independent, but there are epiphytic, endophytic, and saprophytic species. The larger forms and those which grow in tufts or mats are readily recognized in the field. Many of the smaller forms are attached to other water-plants. Drain the water-plants and then squeeze them over a bottle. The sediment is likely to contain a variety of unicellular and other small algæ. Many of the genera are easily kept in the laboratory. Large two-gallon glass jars with ground tops and ground glass covers make convenient aquaria. Put about an inch of pond dirt in some, and a gravel bottom in others. Fill about half full of water. It is a mistake to put too much material into a jar. A wad of *Spirogyra* half as large as one's finger is as much as should be put into a two-gallon jar.

Professor Klebs has shown that various phases in the life-histories of many algæ and fungi may be produced at will. By utilizing his results, the fruiting condition may be induced in many of the common laboratory types. Knop's solution will be needed in most cases. A stock solution which can be diluted as required may be made as follows:

Potassium nitrate, KNO_3	1 g.
Magnesium sulphate, $MgSO_4$	1 g.
Calcium nitrate, $Ca(NO_3)_2$	3 g.
Potassium phosphate, K_2HPO_4	1 g.

Dissolve the first, second, and fourth ingredients in 1 liter of distilled water, and then add the calcium nitrate. A precipitate of calcium phosphate will be formed. For practical purposes

this may be called a 0.6 per cent. solution. Whenever a dilute solution is made from the stock solution, the bottle must be shaken thoroughly in order that a proper amount of the precipitate may be included in the diluted solution. To make a 0.1 per cent. solution, add 5 liters of distilled water to 1 liter of the stock solution; for a 0.3 per cent. solution add 1 liter of distilled water to 1 liter of the stock solution, etc.

The addition of a liter of a 0.2 per cent. solution to 4 or 5 liters of water will often produce a more thrifty growth. Directions for inducing reproductive phases are given in connection with the various types. With a good supply of glass jars, a plenty of Knop's solution, a reasonable control over temperature, and the teacher's usual amount of patience, most laboratory types can be studied in the living condition at all seasons of the year.

Permanent preparations are needed to show details which are not so evident in the fresh material. The unicellular and filamentous members, together with such forms as *Volvox*, are best prepared by the Venetian turpentine method or by the glycerine method. The structure is so much more complicated than in the Cyanophyceae that it demands far more care and skill to make good preparations. Chromo-acetic acid is a good killing and fixing agent for the whole group, but Flemming's fluid (weaker solution) seems to be a little better in some instances. Very good results have been obtained by adding about 3 c.c. of 1 per cent. osmic acid to 100 c.c. of chromo-acetic acid (Schaffner's formula). A formula which gives satisfactory results with *Spirogyra* may cause plasmolysis with *Cladophora*. A few filaments should be placed under the microscope in the fixing agent, and, if plasmolysis occurs, the chromic should be weakened or the acetic strengthened until the suitable proportions are determined. This is a slow process, but difficult forms like *Cladophora* and *Vaucheria* are almost sure to shrink without it. About 24 hours in any of the chromic series and a 2–6 hours' washing in water will be sufficient for members of this group. Only a few of the most commonly studied will be mentioned.

Volvox.—*Volvox* is found in ponds and ditches, and even in shallow puddles. The most favorable place to look for it is in the deeper ponds, lagoons, and ditches which receive an abundance of rain water. *Volvox* is often associated with *Lemna*. It is not easy to keep an abundance of *Volvox* in the laboratory. However, when it disappears, do not throw the culture out, because new cœnobia are likely to develop from the oospores.

For fixing, use chromo-acetic acid with 1 g. chromic acid and 2 c.c. acetic acid to 200 c.c. of water. The addition of 2 c.c. of 1 per cent. osmic acid to 50 c.c. of the above solution may be of some advantage.

The Venetian turpentine method should be used in making mounts of the whole cœnobium. A few broken bits of cover-glass should be placed among the cœnobia to prevent any pressure by the cover. It is hard to mount *Volvox* in glycerine, because the glycerine is likely to run out beyond the cover in such thick mounts. Glycerine jelly is better, but both are inferior to Venetian turpentine in this case, as in most others. For paraffin sections, the material, preferably in sufficient abundance to make a layer half an inch deep in the bottom of a bottle as large as one's finger, is infiltrated with paraffin in the usual way. In imbedding, simply pour the contents of the bottle out so as to form a thin layer on a piece of glass. If a dish is used, the paraffin cake must be very thin. Fig. 26 shows that even such a delicate organism as *Volvox* can be imbedded in paraffin without shrinking.

FIG. 26.—*Volvox*

Photomicrograph of a section stained in Delafield's hæmatoxylin. From a preparation and negative by Dr. W. J. G. Land.

Pleurococcus.—This form, which is used everywhere as a laboratory type of the unicellular green algæ, is found on the bark of trees, where it is more abundant on the north side and near the ground. It is also found on stones and fences, and in moist situations generally. It is easily secured in nearly all localities and at all seasons.

A study of the living material is sufficient for any general course. The bright-green cells, scraped off and mounted in a drop of water, show the rather thick wall, the chromatophores, and usually the nucleus. A drop of iodine will bring out the nucleus, if it does not show already, and will also stain the pyrenoid, if the cell contains one. A mount in Venetian turpentine, stained in Magdala red and anilin blue, shows the nucleus very clearly.

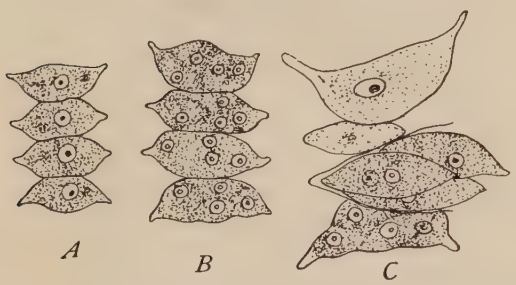

FIG. 27.—*Scenedesmus*

Vegetative condition shown in *A*; successive stages in reproduction shown in *B* and *C*.

Scenedesmus.—*Scenedesmus* (Fig. 27) is found everywhere as a regular constituent of the fresh-water plankton. It is more abundant in stagnant water. It often appears in considerable quantity in laboratory cultures. It may be kept for years in a tightly closed glass jar without renewing the water, the lid being removed only when material is needed.

The form is so small that in living material little more than the general form can be distinguished. Excellent mounts are easily and quickly made. Smear a very thin layer of albumen fixative upon the slide, and add a drop of water containing the *Scenedesmus*. The drop may be inverted for 1 or 2 minutes over the fumes of 1 per cent. osmic acid. No washing is necessary, and good mounts may be made without any fixing whatever. Allow the drop to dry completely. Dip the slide into 95 per cent. alcohol, and then stain in cyanin and erythrosin, or in safranin and gentian-violet. Clear in clove oil and mount in balsam.

Hydrodictyon.—This is popularly known as the "water-net." *Hydrodictyon* is found floating or suspended in ponds, lakes, or slow streams. The young nets are formed within the segments of the older nets. Examine segments 4 or 5 mm. in length for the formation of young nets. The old nets may reach a length of 10 cm. Cultures are easily kept in the laboratory. If material which has been growing in a 0.5 to 1 per cent. Knop's solution be brought into tap water or pond water, zoöspore formation may begin within 24 hours. Nets brought from the nutrient solution into a 1–4 per cent. cane-sugar solution produce zoöspores for a few days.

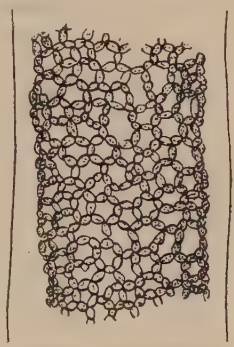

FIG. 28.—*Hydrodictyon*

Zoospores becoming arranged into a new net inside an old segment. Only a small portion of the old segment is shown. Venetian turpentine method. ×125.

Nets of all sizes should be selected for study. The segments are cœnocytic, and the nuclei of the older segments are hard to differentiate, except in stained preparations. Only one nucleus will be found in the young segments, but in the older segments the nuclei become very numerous.

For fixing, use the chromo-acetic solution recommended for *Vaucheria*. The Venetian turpentine method should be used for mounting entire young nets or entire segments of older nets. Magdala red with a rather light stain in anilin blue brings out the nuclei and pyrenoids. For young nets inside the old segments, the blue should be a little deeper. *Hydrodictyon* is easily imbedded and cut. Iron-hæmatoxylin or the safranin, gentian-violet combination are best for paraffin sections. (Fig. 28.)

Ulothrix.—Where the problem of the origin and evolution of sex is studied, *Ulothrix* is an indispensable type. *Ulothrix zonata* is found in springs, brooks, and rivers, occurring in bright green masses attached to stones in riffles, especially in sunny places. It is abundant on stones and piles along the beaches of lakes. Another species is found in stagnant ponds, ditches, and even in watering troughs and rain-barrels. It is difficult to keep in the laboratory the forms which are found in rapidly flowing

water. However, if they are brought in still attached to stones and placed under a stream of tap water, they may live for a couple of weeks and may produce zoöspores every morning. The production of zoöspores may continue for a few days, if the material is merely put into a jar of water; in a 2–4 per cent. cane-sugar solution the production of zoöspores continues a little longer.

While the most instructive study demands living material, some details are more easily seen in stained preparations. Fix in chromo-acetic acid and use the Venetian turpentine method. If it is desirable to stain in iron-hæmatoxylin, stain after the fixing agent has been washed out; then use 10 per cent. glycerine, and after the glycerine has concentrated and has been washed out in 95 per cent. alcohol, transfer to 10 per cent. Venetian turpentine.

Oedogonium.—This form is attached when young, but most species float freely when they are older. Most species are found in quiet waters, especially in ponds and ditches. The floating masses bear some resemblance to *Spirogyra*, but are not so slippery. The best fruiting material is often found attached to twigs, rushes, and various plants, where, to the naked eye, it forms only a fuzzy covering rather than a dense mat.

In studying *Oedogonium diplandrum*, Klebs found that a change from a lower to a higher temperature would induce the production of zoöspores. A culture which had been kept in a cold room with a temperature varying from 6° to 0° C., when brought into a warmer room with a temperature varying from 12° to 16° C., produced an abundance of zoöspores within two days. Light does not seem to have any influence upon the formation of zoöspores in this species, but light is necessary for the formation of antheridia and oögonia. Any culture solutions must be very weak. Sterile material sometimes fruits when brought into the laboratory and placed in open jars with plenty of water and not too much light.

Fix in chromo-acetic acid and use the Venetian turpentine method. Iron-hæmatoxylin or Mayer's hæm-alum is good for staining antheridia, but since these are aqueous stains, the material must be stained after washing in water. The material may then

be got into Venetian turpentine, as directed for *Ulothrix*. Alum carmine is good for the caps, but this must also be treated as an aqueous stain. (Fig. 29.)

Coleochaete.—*Coleochaete* is epiphytic upon the stems and leaves of submerged plants. *C. scutata*, which is the most common species, has a flat, orbicular thallus generally less than 1 mm. in diameter. Some species have a hemispherical form.

For most purposes it is better to mount the whole plant. Complete the staining before trying to remove the *Coleochaete* from its host. Delafield's hæmatoxylin is a good stain. Test the staining by removing single specimens and examining them under the microscope. When the staining is satisfactory, remove the plants with a very sharp scalpel and pass them through the alcohols, 35, 50, 70, 85, 95, and 100 per cent., allowing each to act for half an hour. Clear in clove oil and mount in balsam. The plants may be removed before fixing or at any stage in the process, but they are so small that great care must be taken not to lose them when changing solutions.

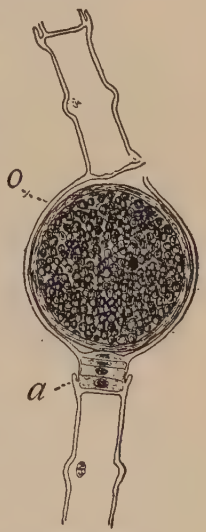

FIG. 29.—*Oedogonium nodulosum*

a, antheridia. *o*, oögonium. Drawn from material fixed in 1 per cent. chromic acid, and stained in Mayer's hæm-alum.

Cladophora.—This genus is found in both salt and fresh water. The fresh-water forms are usually attached to sticks or stones in quiet or running water. The mats feel rough and crisp and, even under a pocket lens, show the characteristic branching by which the form is easily recognized. The absence of a mucous coat makes *Cladophora* a convenient host, for numerous parasitic algæ, among which diatoms belonging to the genera *Cocconeis* and *Gomphonema* are particularly abundant.

For laboratory cultures, select the forms found in quiet water. In fixing, use rather weak solutions. A chromo-acetic acid solution with 1 g. of chromic acid and 4 c.c. of glacial acetic acid to 400 c.c. of water will usually produce no plasmolysis, and will fix the material in 24 hours. After washing in water, stain some of the

material in iron-hæmatoxylin and put it into 10 per cent. glycerine. Also try the Venetian turpentine method, using Magdala red and anilin blue. A rather deep stain in the anilin blue brings out the plate-like chromatophores, each with its single pyrenoid, or the reticular chromatophore with its numerous pyrenoids. In making mounts, select portions showing both vegetative and reproductive phases. (Fig. 30.)

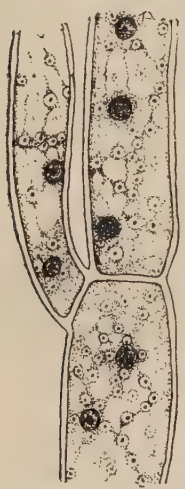

FIG. 30.—*Cladophora* Fixed in chromo-acetic acid, stained in Haidenhain's iron alum-hæmatoxylin.

Diatoms.— Living diatoms are often found clinging in great numbers to filamentous algæ, or forming gelatinous masses on various submerged plants. It is difficult to get really good preparations showing the nucleus and chromatophores. If the diatoms are clinging to filamentous algæ, the algæ with the diatoms attached may be put into chromo-acetic acid (24 hours), washed in water, stained, passed up through the alcohols, allowing each grade to act for half an hour, and then cleared in xylol, or, better, in clove oil or bergamot oil, which do not dry up so rapidly. Here the diatoms may be picked or scraped off from the other algæ, which will probably have become much shrunken by this treatment. Mount in balsam. Haidenhain's iron alum-hæmatoxylin is recommended for the nucleus and the centrosome, which is quite prominent in diatoms. Delafield's hæmatoxylin and erythrosin give a good view of the nucleus and chromatophore.

When the material is in gelatinous masses, it may be fixed in chromo-acetic acid and imbedded in paraffin. There will, of course, be some difficulty in cutting, and many frustules will be broken, but there will, nevertheless, be occasional views which show details better than when the diatoms are mounted whole.

The silicious shells of diatoms are among the most beautiful objects which could be examined with the microscope (Fig. 31). To obtain perfectly clean mounts requires considerable time and patience, but when the material is once cleaned, preparations may

be made at any time with very little trouble. Diatom enthusiasts have devised numerous methods for cleaning diatoms, and separating the various forms from each other, but we shall give here only a few simple, practical methods.

Material for mounts of frustules of living forms "may be obtained by skimming off the brownish scum found on ponds, by squeezing out water weeds, by scraping sticks and stones which are covered at high water, or from the mud of filter beds at pumping-works, or in other places. The material is put in a dish of water, and after it has settled the water is decanted. This is repeated until the water will clear in about one-half hour. The sediment is then treated with an equal bulk of sulphuric acid, after which bichromate of potash is added until all action ceases. After a couple of hours the acid is washed out. To separate the diatoms, place the sediment in a glass dish with water, and when the water becomes clear give the dish a slight rotary motion. This will bring the diatoms to the top, when they may be removed with a pipette and placed in alcohol. To mount, place a number in distilled water, evaporate a few drops of the mixture on a cover-glass, which is then mounted on a slide in balsam."[1]

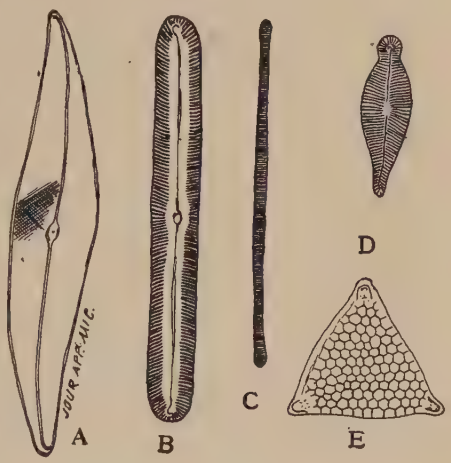

FIG. 31.—Diatoms. × 255

A, Pleurosigma angulatum. B, Navicula dactylis. C, Synedra biceps. D, Gomphonema sphaerophorum. E, Triceratium sp.

Many scouring soaps and silver polishes contain large quantities of fossil diatoms, and the diatomaceous earths are particularly rich. Break up a small lump of such material and boil it in hydrochloric acid. A test-tube is very convenient for this process. Let the diatoms settle, pour off the acid, and then wash in water.

[1] From a review of Dr. Wood's paper on "Diatoms," *Journal of Applied Microscopy*, March, 1899.

As soon as the diatoms settle, the water should be poured off. The washing should be continued until the hydrochloric acid has been removed. When the washing is complete, pour on a little absolute alcohol, and after a few minutes pour off the alcohol and add equal parts of turpentine and carbolic acid. The material will keep indefinitely in this condition, and may be mounted in balsam at any time. In making a mount, put a little of the material on a slide and allow it to become dry, or nearly dry, and then add the balsam and cover. If the balsam should be added too soon, the diatoms are likely to move to the edge of the cover.

Desmids.—The desmids are unicellular, free-floating or suspended algæ. They are much more abundant in soft water than in hard. Deep pools, quiet ponds, and quiet margins of small lakes are good collecting grounds. Collections of other fresh-water algæ often contain some desmids. It frequently happens that a single desirable desmid appears when examining field collections. In such a case, remove it with a fine pipette, and get it into a drop of water on a clean slide, invert it over a bottle of 1 per cent. osmic acid for 2 minutes, leave the slide exposed to the air until almost all the water has evaporated, and then add a drop of 10 per cent. glycerine. In a few hours (6–24) put on a cover and seal. It requires more time, care, and patience than it is worth to attempt staining in such a case.

Sometimes desmids occur in great abundance. They may then be treated like the filamentous algæ, except that more care must be taken not to lose them when changing fluids. The Venetian turpentine method, with Magdala red and anilin blue, will give beautiful preparations. A deep stain with Magdala red and a rather light stain with anilin blue is better for the pyrenoids and nucleus, while a light stain in the red and a deep stain in blue is better for the chromatophores. When the material is sufficiently abundant, paraffin sections may be made as directed for *Volvox*. (Fig. 32.)

Zygnema.—*Zygnema* is one of the commonest algæ of the ponds, swamps, and ditches. The mats are very slippery to the touch. In the field it resembles *Spirogyra*, but is distinguished

by the two characteristic chromatophores which are readily seen with a good pocket lens. Sometimes conjugation can be induced by bringing the material into the laboratory and placing it in open jars with plenty of water and not too much light.

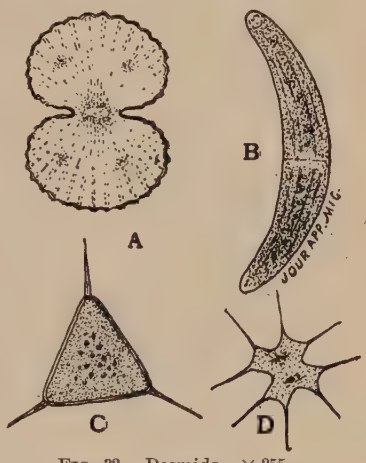

FIG. 32.—Desmids. × 255

From glycerine preparations. Not stained. *A*, *Cosmarium pectinoides*. *B*, *Closterium cucumis*. *C*, *Staurastrum cornutum*. *D*, *Arthrodesmus octocornis*.

Iron-hæmatoxylin is a good stain for conjugating material. The stain should be extracted until the four chromatophores become distinct. The nuclei are small and inconspicuous. It is better, even when using aqueous stains, to get the material into Venetian turpentine. The chromatophores do not stain as readily as those of *Spirogyra*, and consequently it is necessary to use stronger stains or more prolonged periods.

FIG. 33.—*Zygnema*

The filament at the left shows three zygospores and one parthenogenetic spore which is distinguished by having only two chromatophores. The filament on the right shows two cells, each with a pair of stellate chromatophores. Drawn from material fixed in 2 per cent. formalin and stained in iron alum-hæmatoxylin.

Methods, in general, are the same as for *Spirogyra* (Fig. 33).

Spirogyra.—Probably no alga has been more studied by pupils, teachers, and investigators than *Spirogyra* (Fig. 34). Nearly all of the numerous species belong to the low, quiet waters of ponds and ditches, where they often form large, flocculent green mats nearly covering the surface of the water. A few species occur in running water. The mats are very slippery to the touch—a character which helps to recognize the genus in the field. In the larger species the characteristic spiral chromatophore can be seen with a good pocket lens, thus completing the identification, as far as the genus is concerned.

Mats in which zygospores have been formed are likely to show a pale, or even a brownish, color, due to the brownish walls of the zygospores. This color, however, is not always, or even usually, due

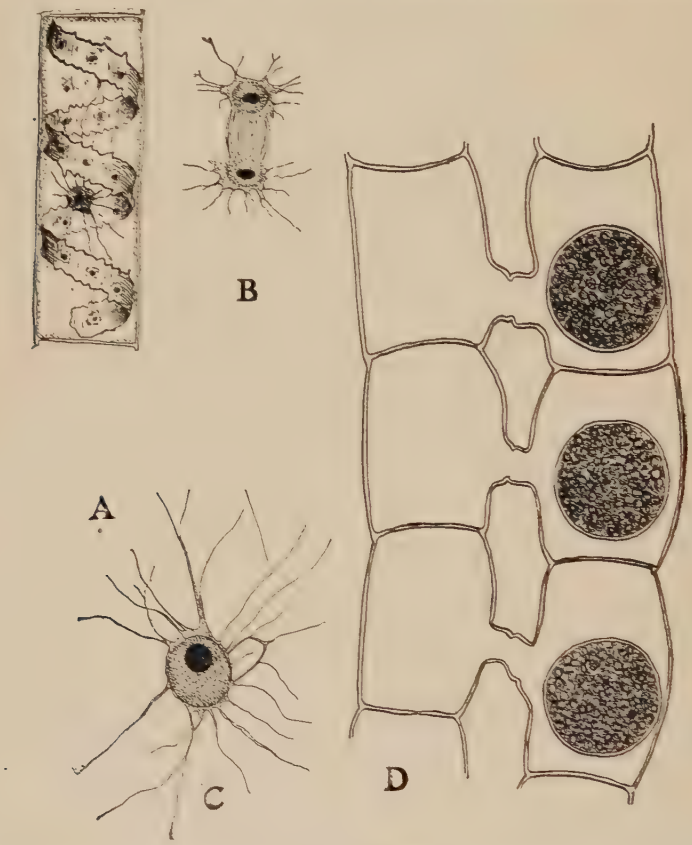

FIG. 34.—*Spirogyra*

From material fixed in chromo-acetic acid and stained in iron alum-hæmatoxylin. *A*, single cell showing nucleus, chromatophore, and pyrenoids. *B*, a nucleus undergoing division. *C*, a resting nucleus. *D*, zygospores, each showing two nuclei.

to zygospores, but is more often due to the death and degeneration of the plants. Mats in early stages of conjugation and those with zygospores show as bright a green as vigorously growing material.

Spirogyra is not easy to keep in the laboratory. The small species keep better than the larger ones. Put only a small amount

of the material in a jar and use rain water. If necessary to use tap water, let the water run for a minute before taking the water for the culture. Most metals are poisonous to *Spirogyra*, even the small amount taken up by the water while standing in the water pipe being detrimental.

The species found in running water will usually conjugate within a week when brought into the laboratory and placed in rain water or tap water. Species belonging to quiet waters, when brought into the laboratory and placed in a 0.2 Knop's solution, are likely to undergo rapid cell division and growth. After the alga has remained in such a culture for a few days or for a week, conjugation may be induced by transferring to rain water or tap water, and keeping the culture in bright sunlight. Conjugation may begin within 3 or 4 days. Variations in temperature between 15° and 25° C. have little influence upon conjugation.

The following is a good fixing agent for most species of *Spirogyra:*

Chromic acid	1 g.
Glacial acetic acid	4 c.c.
Water	400 c.c.

The fixing is probably sufficiently complete within 6 or 8 hours, but it does no harm to leave material in the fixing agent over night or for 24 hours. A mat half as large as one's hand will be washed thoroughly in 2 or 3 hours, if put into a gallon of lukewarm water. The water should be changed once or twice. Directions for staining are given in the chapter on "The Venetian Turpentine Method."

The paraffin method should be used for mitotic figures, although these appear fairly well when the filaments are stained in Magdala red and anilin blue and mounted whole by the Venetian turpentine method.

Vaucheria.—This form can always be obtained in greenhouses, especially in the fernery, where it forms a green felt on the pots. The greenhouse form is likely to be *Vaucheria sessilis*. Another species, *V. geminata*, is very common in the spring, when it may be found in ponds and ditches. (Fig. 35.) *Vaucheria* is also found in running water, but in this situation is almost certain to be sterile.

In the vicinity of Chicago, *V. geminata* appears late in March or early in April and within a few weeks begins to fruit abundantly. The fruiting continues for 4 to 8 weeks, and then the alga may disappear until later in the season, when some of the oöspores germinate.

Vaucheria sessilis is found at all seasons in the greenhouses, but it is usually in the vegetative condition. Klebs found that the formation of oögonia and antheridia can be induced in *V. repens* (a variety of *V. sessilis*) within 4 or 5 days by putting the material into a 2–4 per cent. cane-sugar solution in bright sunlight. The sex organs will not be formed in weak light or in darkness.

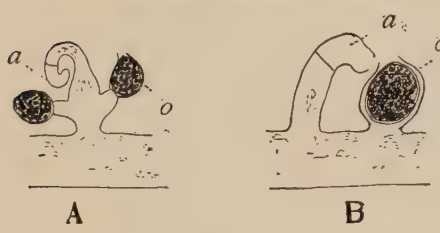

FIG. 35.— *Vaucheria*

A, *Vaucheria geminata*. *B*, *V. sessilis*. *a*, antheridia. *o*, oögonia.

The formation of zoöspores may be induced in the following way: Cultivate in a 0.1 to 0.2 per cent. Knop's solution for a week, then bring the material into tap water, and keep the culture in the dark. Zoöspores may appear within 2 days. Bright light or a temperature higher than 15° C. will check the production of zoöspores. A 2 per cent. cane-sugar solution kept in the dark is also likely to furnish zoösporic material. If no zoöspores are formed when the solution is kept in the dark, the nutrition has been too weak: strengthen the nutrient solution and keep the culture in the light for a few days; then put the culture in the dark, and zoöspores should appear. The formation of zoöspores may continue for a couple of weeks.

Aplanospores of *V. geminata* are formed in nature when the plant is growing upon damp ground. The aplanospores may also appear in a 4 per cent. cane-sugar solution.

In fresh 0.5 Knop's solution in bright light, cultures remain in the vegetative condition, and the result is the same in weak light, if the nutrient solutions are seldom changed. Such cultures may be kept indefinitely by changing the nutrient solution whenever a whitish scum appears on the surface.

Fixing solutions which are successful with *Spirogyra* may prove ruinous to *Vaucheria*. In general, fixing solutions for

Vaucheria should be very weak. Mount a few filaments, and with a pipette add a few drops of the fixing agent. Plasmolysis is likely to take place within 10–30 seconds, if it is to take place at all. If plasmolysis takes place, weaken the fixing agent. The following formula has given the best results with *V. geminata:*

Chromic acid	1 g.
Glacial acetic acid	8 c.c.
Water	800 c.c.

This is a very weak solution. A loose mat as large as one's finger requires 100 c.c. of the reagent, and about 24 hours is necessary for fixing. Such a piece of material is washed thoroughly in two hours, if placed in a gallon of warm water. The water need not be changed more than once or twice. After washing, place in 10 per cent. glycerine and follow the Venetian turpentine method. With Magdala red and anilin blue the oöspores will show various shades of red, according to the stage of development; the filaments will show various mixtures of blue and red.

If the material is to be imbedded in paraffin, it will be safer, and will save time, to put it into 10 per cent. glycerine after the washing in water. When the glycerine has concentrated, wash it out with 95 per cent. alcohol, dehydrate in absolute alcohol, and proceed as usual.

Chara.—*Chara* is found in ponds, lagoons, and ditches. Once seen, it is always readily recognized (Fig. 36). In the ponds and lagoons along the southern shores of Lake Michigan it fruits so abundantly that the whole pond shows an orange color due to the immense numbers of antheridia. In the lagoons of the Chicago parks *Chara* is so abundant that it must be dredged out every summer.

Chara is easily kept alive throughout the year in the laboratory. A two-gallon glass jar with an inch of pond dirt, sand, and gravel at the bottom, and nearly filled with tap water, is all that is needed for a successful culture. If the jar is to be covered, it should not be more than two-thirds full of water. Not more than a dozen plants should be put into such a jar.

A rather strong solution should be used for fixing. The following will give good results:

Chromic acid	1 g.
Glacial acetic	1 c.c.
Water	100 c.c.

In about 24 hours this not only fixes, but it dissolves the lime with which most species are coated.

For paraffin sections select the tip of the plant, a piece about half an inch in length. Sections of this may show, not only the large apical cell, but also various stages in the development of antheridia and oögonia. Delafield's hæmatoxylin is a very good stain for the apical cell and for the development of antheridia and oögonia. The later stages in the development of antherozoids are brought out more clearly by the safranin, gentian-violet, orange, or by cyanin and erythrosin, but here unusual care must be taken not to stain too deeply.

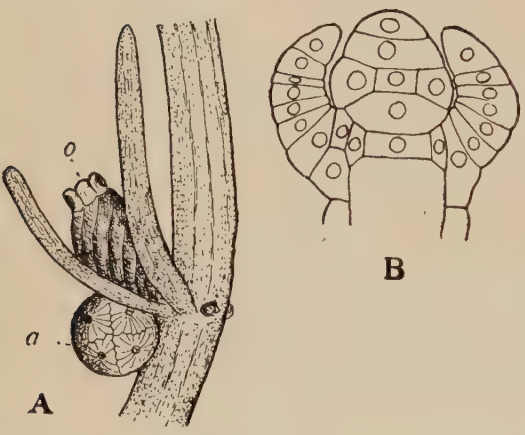

FIG. 36.—*Chara*

A, portion of a branch showing an antheridium, *a*, and an oögonium, *o*. × 35. *B*, median longitudinal section of an apical cell. Drawn from a preparation fixed in chromo-acetic acid, and stained in Delafield's hæmatoxylin. × 225.

The antheridium of *Chara* stains so rapidly that the beginner uniformly makes poor preparations. In order to get good preparations of the antheridium, it is necessary to disregard other structures, which will be stained lightly or not at all when the stain is just right in the antheridial filaments.

If it is desired to mount whole branches showing the antheridium and oögonium in position, as in Fig. 36, use the Venetian turpentine method, staining in Magdala red alone, or in Magdala red and anilin blue. Good mounts showing shield, manubrium, capitula, and filaments may be obtained by crushing an antheridium under a cover-glass. For this it is better to stain in Magdala red alone, since any overstaining is easily corrected by exposing the preparation to direct sunlight.

CHAPTER XIV

PHAEOPHYCEAE AND RHODOPHYCEAE

PHAEOPHYCEAE. BROWN ALGÆ

The Phaeophyceae are almost exclusively marine, including very delicate and also very coarse forms. Chromo-acetic acid is a good fixing agent.

The following formula of Flemming, used by Dr. Davis at the Marine Biological Laboratory at Woods Hole, gives good results with delicate filamentous brown algæ, such as *Ectocarpus*, as well as for the larger types like *Fucus*, *Sargassum*, and *Laminaria*.

1 per cent. chromic acid in sea water	25 c.c.
1 per cent. acetic acid in sea water	10 c.c.
Sea water	65 c.c.

Unless there are stock solutions of the 1 per cent. chromic acid and the 1 per cent. acetic acid, the following is a more convenient method for making the reagent:

Chromic acid	1 g.
Glacial acetic acid	0.4 c.c.
Sea water	400 c.c.

Material should be left 12–24 hours in the fixing fluid, and should then be washed for 3 or 4 hours in sea-water. The texture of the brown algæ is generally so firm that material may be washed in a running stream of water.

After washing, pass through the alcohols up to 70 per cent. alcohol, where the material may be kept. The lower alcohols should be changed a few times to get rid of the sea water.

According to Dr. Davis, the details of nuclear structure and mitotic phenomena are more satisfactory in material fixed in Flemming's weaker solution, or even better in a solution with the following proportions:

1 per cent. chromic acid in sea water .	25 c.c.
1 per cent. acetic acid	10 c.c.
1 per cent. osmic acid	5 c.c.
Sea water	60 c.c.

The blackening due to the osmic acid must be removed with peroxide of hydrogen before sections are stained.

Sphacelaria.—The apical cell affords an excellent study of the structure of protoplasm. During mitosis the centrosomes and the kinoplasmic structures are quite prominent. For these features it is a good plan to break off the tips so as to have only pieces one-fourth or one-half an inch long, which will lie flat in the paraffin. The tips should be broken off after the material has been brought into xylol. If whole tufts are imbedded, the branches diverge enough to make perfectly longitudinal sections of the apical cells rather rare. Iron-hæmatoxylin with a faint staining in orange is a satisfactory combination.

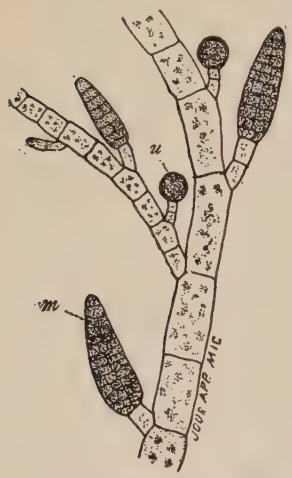

FIG. 37.—*Ectocarpus confervoides*

From a preparation stained in Mayer's hæm-alum, and mounted in glycerine. × 255. *m*, multilocular sporangium. *u*, unilocular sporangium.

Ectocarpus.—Branches may be mounted whole in Venetian turpentine or in glycerine. To mount in Venetian turpentine, place the material in 10 per cent. glycerine after the fixing agent has been washed out with sea water, and proceed as usual. In dealing with material which has been standing in 70 per cent. alcohol, transfer it to 85 per cent. alcohol, and after half an hour, stain in Magdala red and anilin blue, and proceed as usual.

To mount fresh material in glycerine or glycerine jelly, stain in iron-hæmatoxylin or in eosin after the fixing agent has been washed out. Then transfer to 10 per cent. glycerine. (Fig. 37.)

Laminaria.—In forms like this, small portions should be cut out with a razor and then placed in the fixing agent. Iron-hæmatoxylin seems to be the most satisfactory stain.

Material for habit work should be allowed to dry. When needed for use, it is put into water until it can be handled without breaking. It can be used repeatedly. Other coarse forms may be treated in the same way.

Fucus.—Material for habit work may be dried as in the case of *Laminaria*. For the growing points and conceptacles, small pieces should be cut off with a razor. If the fruiting tips be cut through lengthwise before they are cut off, the fixing will be more satisfactory.

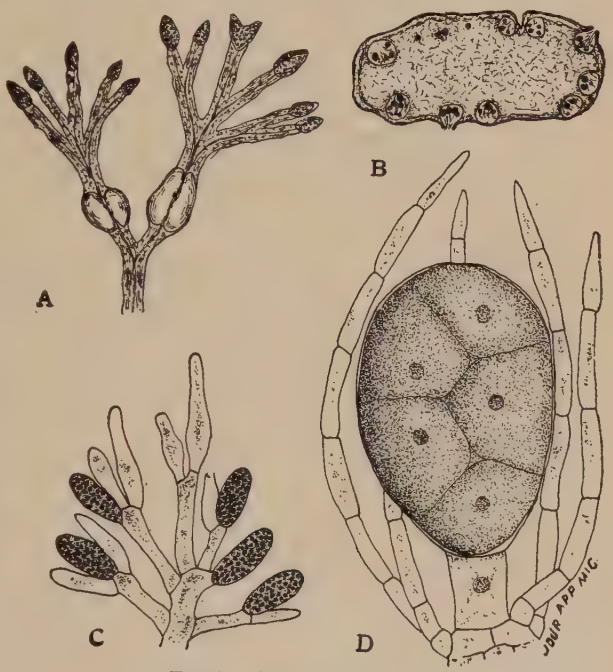

FIG. 38.—*Fucus vesiculosus*

A, small portion of plant showing bladders and fruiting branches. One-half natural size. *B*, transverse section of fruiting branch showing oögonial conceptacles. × 6. *C*, antheridia and paraphyses. From a preparation fixed in chromo-acetic acid, stained in borax carmine, teased out and mounted in balsam. × 255. *D*, oögonium showing five of the eight oöspheres. Prepared as in *C*.

Fertilization occurs at all seasons, but autumn is the most favorable time for collecting this stage and the early stages in the division of the fertilized egg. The eggs and sperms form slimy masses, the antheridia being orange-red, and that containing oöspheres a dirty green. Bring both kinds of slime to the laboratory and mix a drop of the red with a drop of the green. The movements of the egg can be observed, and material for a study of fertilization and later stages is easily secured. In fixing fertilization and succeeding stages, it is worth while to use some of the regular Flemming's weaker solution, as well as the solution without the osmic acid.

For sections of the conceptacles it is better not to cut across the whole tip, but to cut off pieces of the rind containing half a dozen conceptacles. Such pieces are more easily imbedded and cut. There is no difficulty in cutting such pieces in paraffin. Iron-hæmatoxylin is a good stain. Safranin and gentian-violet are also satisfactory, but care must be taken not to overstain, since *Fucus* usually stains deeply and rapidly.

For such views as are represented in Fig. 38, *C* and *D*, the material should be stained in bulk in borax carmine or alum carmine. The process for borax carmine is as follows:

1. Borax carmine, 24 hours.
2. Acid alcohol (2 drops of HCl in 50 c.c. of 70 per cent. alcohol), until the color becomes a clear red. This may take an hour or even a day.
3. 70 to 100 per cent. alcohol, 2 hours each.
4. Clear in cedar oil, bergamot oil, or oil of cloves.
5. Tease out the contents of the conceptacles sufficiently to show details, and mount in balsam.

The process for alum carmine is the same, except that no acid alcohol is used.

It would be worth while to try the Venetian turpentine method for views like those shown in *C* and *D* of Fig. 38.

RHODOPHYCEAE. RED ALGÆ

The red algæ belong almost exclusively to salt water, but a few genera are found only in fresh water, usually in running water, and a few forms occur both in salt and in fresh water.

The technique is more difficult than in the case of the brown algæ. Until something better is suggested, the same method of fixing and washing may be used as for the brown algæ, except that in the case of the few fresh-water forms, fresh water should be used in making the fixing agent and in washing it out. For *Polysiphonia*, and doubtless for many other forms, the period in the fixing agent should be very much shortened. Picric acid, corrosive sublimate, and absolute alcohol have been tried, but do not give as good results as the chromo-acetic acid or Flemming's fluid.

Batrachospermum.—This is a green, fresh-water member of the red algæ. It is not very uncommon in small streams. (Fig. 39.)

The cells are so small that it is hardly worth while to attempt sectioning them. Very good preparations showing the nuclei may be obtained by staining in Mayer's hæm-alum, or Haidenhain's iron-hæmatoxylin. After the material is in glycerine ready for mounting, tease out a small portion, and still further dissociate the filaments by tapping smartly on the cover.

Material stained in eosin shows the external structure well, but may not bring out the nuclei.

The Venetian turpentine method might be more satisfactory.

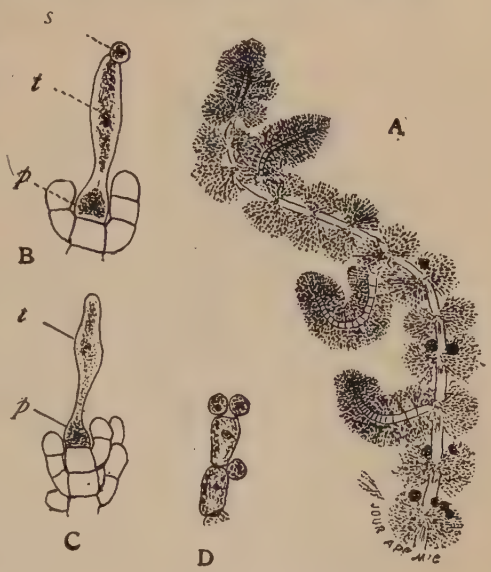

FIG. 39.— *Batrachospermum moniliforme*

From a preparation stained in Mayer's hæmalum and mounted in glycerine. *A*, portion of plant showing branches and several cystocarps; × 25. *B*, a procarpic branch showing carpogonium (*p*), and trichogyne (*t*), with an antherozoid (*s*) attached. × 255. *C*, a younger branch showing carpogonium and trichogyne. × 255. *D*, branch with three antherozoids. × 255.

Nemalion.— Methods for preparing *Nemalion* have been described by Wolfe.[1] Chromo-acetic acid proved to be most satisfactory for fixing. For studying fertilization, mounts were made as follows: "Young tips were crushed in water under a cover-glass and on a slide that had previously been treated with fixative; the cover was then removed, and the water on the slide allowed to evaporate. The gelatinous nature of the wall prevents the contents of the cell from being affected by this treatment even when the albumen has hardened sufficiently to hold the filaments firmly in place." Stain in safranin and gentian-violet, and mount in balsam.

Iron-hæmatoxylin is recommended for paraffin sections. The sections must be very thin, 5 μ or less.

[1] Wolfe, James J., "Cytological Studies in Nemalion," *Annals of Botany*, **18**: 607-630, 1904.

"Material killed in 2 per cent. formalin in sea water and gradually transferred to pure glycerine kept its color perfectly."

The Venetian turpentine method gives good results even when tried with material which has been standing in 70 per cent. alcohol. In applying the Venetian turpentine method to such material, transfer to 85 per cent. alcohol, stain, and then transfer to 10 per cent. Venetian turpentine. The method would doubtless give better satisfaction with fresh material.

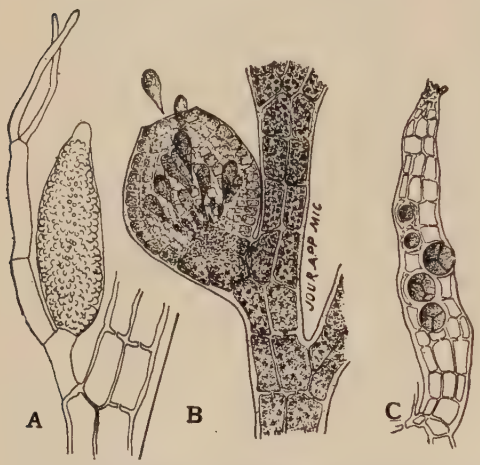

FIG. 40.—*Polysiphonia fibrillosa*

From a preparation fixed in chromo-acetic acid, stained in eosin, and mounted in glycerine. × 255. *A*, an antheridium. *B*, a cystocarp with carpospores. *C*, tetrasporic branch with tetraspores.

The glycerine method can also be used with alcoholic material. Get the material down gradually from 70 per cent. alcohol to water, stain in iron-hæmatoxylin or in eosin, and then place in 10 per cent. glycerine. Mount small pieces with not too much glycerine, and then tap smartly on the cover until the filaments are sufficiently dissociated.

Polysiphonia.—This is a difficult form to handle. Dr. Davis finds that material of *Polysiphonia* should be left in a weak chromo-acetic acid, after the formula given for the brown algæ, not longer than 5 or 10 minutes. Wash carefully in a gentle stream of sea water, and then pass gradually up to 70 per cent. alcohol. With very delicate forms, like *Callithamnion* and *Griffithsia*, the washing may be in part or even wholly omitted, and the chromic acid extracted by the lower alcohols, the material being kept in the dark.

For glycerine mounts, alcoholic material must be passed down through the alcohols to water, after which it may be stained in iron-hæmatoxylin or in eosin, and then got into glycerine in the usual way (Fig. 40).

In making mounts in Venetian turpentine from alcoholic material, transfer to 85 per cent. alcohol, stain, and then transfer to the 10 per cent. Venetian turpentine.

Doubtless both the glycerine mounts and the Venetian turpentine mounts would be more satisfactory if made from fresh material.

Corallina.—The Corallines, whose surface is incrusted with lime, require a fixing agent much stronger in chromic acid. The following formula is recommended by Dr. Davis:

1 per cent. chromic acid in sea water .	60 c.c.
1 per cent. acetic acid in sea water . .	40 c.c.

The fixing agent should be changed several times. Material should remain in the fixing agent several hours after the lime has been entirely dissolved. Nuclear phenomena are more satisfactory when some osmic acid has been added to the fixing agent. Such material must be bleached in peroxide of hydrogen before staining.

CHAPTER XV

FUNGI

In general, the filamentous fungi are treated like the filamentous algæ, while the fleshy forms are cut in paraffin. Bacteriological methods are used in making test-tube and Petri dish cultures. Professor Klebs's investigations make it easy to secure material of many forms in various phases of their life-histories.

PHYCOMYCETES

Mucor.—This familiar mold appears with great regularity on bread. The following is a sure and rapid method for obtaining *Mucor:* Place a glass tumbler in a plate of water, put on the tumbler a slice of bread which has been exposed to the air for a day, and cover with a glass jar. The bread must not become too wet.

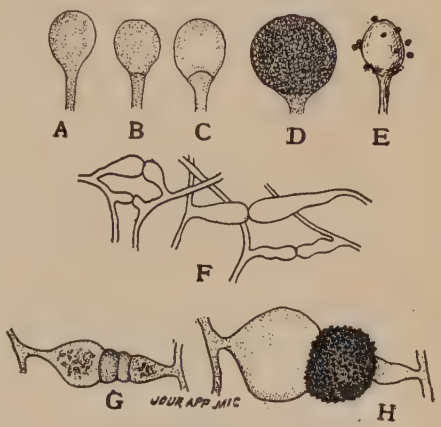

FIG. 41.—*Mucor stolonifer.* × 255

A–D, successive stages in the development of the sporangium. Drawn from living material. *E*, columella with a few spores adhering. *F–H*, stages in the formation of the zygospore. From a preparation fixed in corrosive sublimate, stained in Delafield's hæmatoxylin, and mounted in glycerine.

To obtain such a series as is shown in *A–D* of Fig. 41, the material should be studied before the sporangia begin to turn black. Such a study is most satisfactory with fresh material, and since fresh material is always available, it is hardly worth while to make preparations. It is not difficult, however, to make preparations.

Material may be fixed in chromo-acetic acid (1 g. chromic acid and 2 c.c. glacial acetic acid to 200 c.c. of water). Fix for 10–24 hours, wash in water, and mount by the Venetian turpentine method or by the glycerine method.

Corrosive sublimate and acetic acid can also be recommended:

Corrosive sublimate ($HgCl_2$) . . .	2 g.
Glacial acetic acid	2 c.c.
Water	100 c.c.

Use hot (85° C.). Material will be fixed in 10–20 seconds. Wash in water, using iodine as directed for washing out corrosive sublimate.

For paraffin sections, the addition of 2–3 c.c. of 1 per cent. osmic acid to 50 c.c. of chromo-acetic solution may give better results.

The phase in the life-history represented in *F–H* of Fig. 41 is rarely met in nature or in cultures. The recent researches of Dr. Blakeslee show why zygospores are so infrequent. The conjugating filaments belong to different strains of mycelia which, for convenience, may be called male and female mycelia. When male and female mycelia come together, zygospores are formed. In attempting to get zygosporic material, make a large number of cultures and mix the mycelia; also make new cultures by sowing upon one piece of bread the spores from many cultures. In any case, sporangia are likely to be developed for a while before zygospores appear. The zygosporic material is first recognized by the naked eye by the yellowish color due to the oil in the large swollen suspensors. Glycerine mounts without any staining give excellent views. A very light stain in Delafield's hæmatoxylin brings out the mycelium. The Venetian turpentine method, with or without any staining, can be recommended.

Professor Klebs had no success in trying to induce this condition in *Mucor*.

In the related genus, *Sporodinia*, which is rather common in summer upon fleshy fungi, especially upon *Boletus* and its allies, the zygosporic condition is not infrequent. The very damp atmosphere and the nutrition necessary for the formation of zygospores may be provided in the laboratory in the following way: Put a little water in a glass battery jar and place filter paper around the inside of the jar so that it will take up water and so keep the sides of the jar moist. Place a small beaker or

dish, without any water in it, in the bottom of the jar, and in the beaker place a small piece of bread dampened with the juice of prunes. Infect the bread with spores, or use a piece of bread upon which mycelium is already growing. Sections of the root of *Daucus carota* may be used instead of the bread. Put a piece of wet filter paper on a pane of glass and cover the jar. Begin to examine after 24 hours. The zygospores may appear in 4 or 5 days. A very full account of the methods by which the various phases of the life-history of *Sporodinia* may be produced at will is given by Klebs in the *Jahrbücher für wissenschaftliche Botanik* **32**: 1–69, 1898.

Saprolegnia.—This is an aquatic mold, very common upon insects and fishes. Cultures are easily and quickly made. Bring in a quart of water from any stagnant pond or ditch, and into the water throw a few flies. After 12–24 hours throw the water away, rinse the flies in clean water, and put them into tap water. Sporangia will probably appear within 24 hours. The water must be changed every day to keep bacteria from ruining the culture. Small pieces of fresh beef, not more than 2 mm. square, or small pieces of boiled white of egg are better than flies, if sections are to be cut. Sporangia may be produced in the greatest abundance by cultivating the mycelium for several days and then transferring it to pure water or to distilled water. As long as the nutrient solution is sufficiently strong and fresh, only sterile mycelium will be produced.

To secure oösporic material, mycelium which has been highly nourished for several days in a nutrient solution is brought into a 0.1 per cent. solution of leucin, or into a 0.05 to 0.1 per cent. solution of hæmoglobin. Begin to examine after 24 hours.

Satisfactory material for general laboratory purposes can be secured as just described. Absolutely pure cultures can be secured only by observing all the precautions necessary in bacteriological work.

Albugo.—This fungus is quite common on Cruciferae, where the white "blisters" or "white rust" form quite conspicuous patches.

Affected portions of leaves and stems should be fixed in chromo-acetic acid and cut in paraffin. Sections 5 μ or less in thickness will be found most satisfactory. Safranin, gentian-violet, orange seems to be the best stain for differentiating the nuclei. (Fig. 42.)

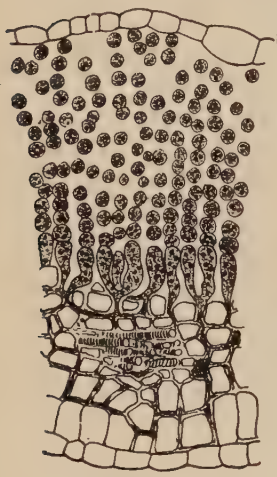

FIG. 42.—*Albugo candidus* on *Capsella*

Trans. sec. of a blister on the leaf. × 255. From a preparation fixed in Flemming's fluid and stained in safranin, gentian-violet, orange.

It is more difficult to get good sections of the plant in the oösporic condition. The oösporic phase of *Cystopus bliti* is easily recognized on *Amarantus*, where the oöspores may be seen with the naked eye by holding the leaf up to the light. The oöspores usually occur in more or less circular patches upon the leaf. When they occur among the floral structure, there is often a slight reddish coloration. Unfortunately for the collector, it is very seldom that any red coloration in *Amarantus* is due to the desired material.

To show the structure of oöspheres and antheridia, sections must not be thicker than 5 μ. Sections as thick as 10–15 μ may be cut to show the position of oögonia and antheridia, although such sections are thick to give satisfactory views of the nuclei.

HEMIASCOMYCETES

Saccharomyces.—Until somewhat recently it was considered rather difficult to demonstrate the nucleus of the yeast cell. With fresh growing yeast the following method by Wager should be successful: Fix in a saturated aqueous solution of corrosive sublimate for at least 12 hours. Wash successively in water, 30 per cent. alcohol, 70 per cent. alcohol, and methyl alcohol. Place a few drops of alcohol containing the cells on a cover, and when nearly dry add a drop of water. After the yeast cells settle, drain off the water and allow the cells to dry up completely. Place the cover, or slide, with its layer of cells in water for a few

seconds, and then stain with a mixture of fuchsin and methyl green, or fuchsin and methylen blue. Mount in glycerine or in balsam.

ASCOMYCETES

This group, popularly known as the "sac fungi," contains an immense number of saprophytic and parasitic forms. The green mold on cheese and leather, the leaf curl of peach, the black knot of cherry and plum, and the powdery mildews are familiar to everyone. The few objects selected will enable the student to experiment, but he must not be discouraged if success does not crown the first attempt, for some members of the group present real difficulties.

FIG. 43.—*Peziza odorata* Three asci and many paraphyses. × 245. Fixed in corrosive sublimate, stained in bulk in alum carmine. Teased out and mounted in balsam.

Peziza.—The Pezizas and related forms are fleshy, and present but little difficulty in fixing, cutting, or staining. They are abundant in moist places, on decaying wood, or on the ground. The apothecia have the form of little cups, which are sometimes black and sometimes flesh-colored, but often orange, red, or green.

For general morphological work it is better to tease out fresh or preserved material. Such views as that shown in Fig. 43 are easily obtained in this way. For permanent preparations showing such views, it is better to stain in bulk in alum carmine or in Delafield's hæmatoxylin, and then tease out the asci in glycerine or balsam. Sections showing the entire ascus should be 10–15 μ in thickness.

For the free nuclear division in the ascus, and also for the development of the ascospores, Flemming's weaker solution, followed by the safranin, gentian-violet, orange combination has given the best results. Cyanin and erythrosin are also to be recommended. The latter combination stains better when the fixing

agent contains no osmic acid. Sections should be 3 μ in thickness. Sections thicker than 5 μ are unsatisfactory for any study of the cytological features of the ascus.

Eurotium.—*Eurotium*, or *Aspergillus* as it is frequently called, is a very common mold found on bread, cheese, decayed and preserved fruit, etc. In the conidial stage it is green and in the ascosporic stage, yellow, reddish-yellow, or reddish-brown.

Eurotium is almost sure to appear upon bread which is kept moderately moist. First there appears a white, flocculent mycelium, then the conidial stage (Fig. 44), and later the perithecial stage is sometimes found. The perithecial stage is indicated by the yellowish or darker colors, just as the conidial stage is known by the green color.

The conidial stage is so easily obtained, that it is hardly necessary to make cultures. If the bread be wet with a 10 per cent. solution of cane sugar or with grape juice, this stage appears sooner and in greater abundance. A temperature of 22° to 30° C. is also a favorable condition.

The perithecial stage is not found so frequently, but can generally be secured by examining moldy preserves. However, if one has the mycelium or spores, the sexual stage can be induced. Soak a piece of bread in a 20 per cent. solution of grape sugar in grape juice; upon this sow the spores and keep at a temperature of about 28° C. After 4 or 5 days, begin to examine. A 40 per cent. solution of cane sugar in the juice of prunes is also a good nutrient solution.

For class use or for permanent preparations it is best to select rather young material which shows various stages in development, from the swollen end of the hypha to the ripe spore (Fig. 44). Permanent preparations of the conidial stage, as shown in Fig. 44, and also of the coiled twisted filaments which initiate the ascosporic stage, should be made by the Venetian turpentine method or by the glycerine method.

Fix in 1 per cent. chromo-acetic acid (1 g. chromic acid and 1 c.c. acetic acid and 100 c.c. water) for 24 hours; wash in water

2–4 hours; transfer to 10 per cent. glycerine, and continue the Venetian turpentine method.

Material may be fixed in corrosive sublimate acetic acid (corrosive sublimate 2 g., glacial acetic acid 2 c.c., and water 100). Use it hot (85° C.). One minute is long enough. Wash in water and add, a few drops at a time, the iodine solution used in testing for starch. At first, the brownish color caused by the iodine will disappear, but after a certain amount has been added, the brownish color will remain. The material is now ready for the 10 per cent. glycerine if the Venetian turpentine is to be used, or for the eosin or iron-hæmatoxylin, if the glycerine method is preferred.

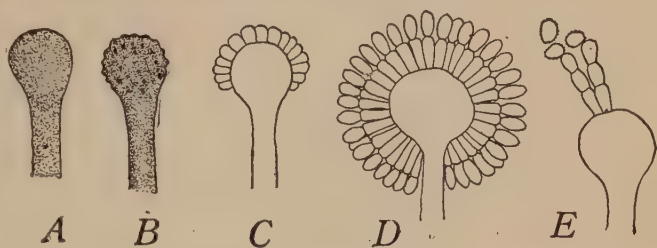

FIG. 44.—*Eurotium* (*Aspergillus*)
From material growing on a hectograph pad. Fixed in chromo-acetic acid, stained in eosin, and mounted in glycerine. *A–E*, successive stages in development. × 375.

A very rapid method for this and for similar, small filamentous forms may be added. Forms as large as *Thamnidium elegans* can be mounted successfully by this method.

1. 100 per cent. alcohol, 2 minutes.
2. Eosin (aqueous), 2 minutes.
3. 1 per cent. acetic acid, 2 to 10 seconds.
4. Wash in water 5 minutes, changing frequently.
5. Mount directly in 50 per cent. glycerine, and seal.

If the material gets through the first four stages without shrinking but collapses at the fifth, put it into 10 per cent. glycerine and allow it to thicken as usual.

All the later perithecial stages are easily caught in paraffin.

Penicillium.—This green mold is found everywhere upon decaying fruit, upon bread, and upon almost any decaying organic substance.

Material is even more easily secured than in case of *Eurotium*,

and *Penicillium* is an easier type for laboratory study. Such satisfactory mounts can be made from the living material that it is hardly worth while to make mounts. The very rapid method described for *Eurotium* will furnish good mounts if permanent preparations are desired.

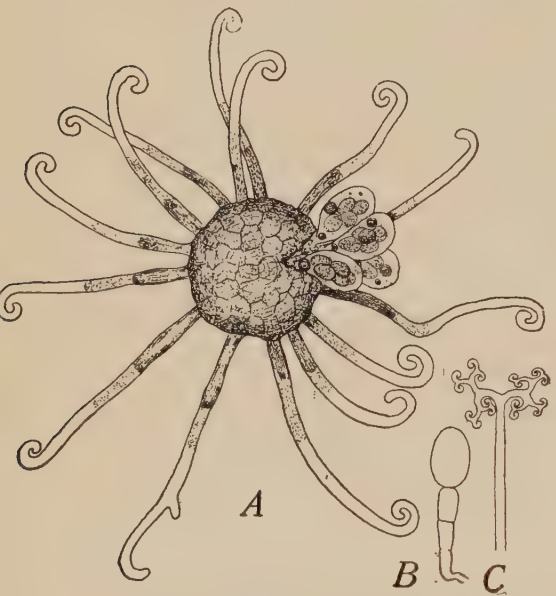

FIG. 45.—*A*, *Uncinula necator* on *Ampelopsis quinquefolia*. × 192. Four asci containing ascospores have been forced out by pressing out the cover. Fixed in hot corrosive sublimate, stained in fuchsin, and mounted in balsam. *B*, a conidiospore; and *C*, an appendage of *Microsphaera alni*, drawn from living material. × 192.

The Erysipheae.—The mildews are found throughout the summer and autumn on the leaves of various plants. Some of the most abundant forms are *Microsphaera alni* on the common lilac; *Sphaerotheca Castagnei* on *Bidens frondosa* and other species, on *Erechtites hieracifolia*, and on *Taraxacum officinale; Uncinula necator* on *Ampelopsis quinquefolia*, and *U. salicis* on *Salix* and *Populus; Erysiphe commune* on *Polygonum aviculare;* and *Erysiphe cichoriacearum* on numerous *Compositae* and *Verbenaceae*. For herbarium purposes they may be preserved by simply drying the leaves under light pressure. When needed for examination the leaf should be soaked in water for a few minutes, after which the perithecia may be scraped off and mounted in water. In mounting great care must be taken not to break off the appendages. The asci may be forced out by pressing smartly on the cover. (Fig. 45.)

For permanent mounts of entire perithecia with appendages, fix in 3 per cent. formalin 24 hours, wash in water 1 hour, stain in aqueous eosin 24 hours, treat with 1 per cent. acetic acid 1 minute, wash thoroughly in water, and then transfer to 10 per

cent. glycerine, which should be allowed to concentrate as usual. If chromic acid, corrosive sublimate, or alcohol be used for fixing, the appendages become brittle and very easily break off. However, the chomo-acetic mixtures are better if it is desired to make paraffin sections showing the development of the perithecium with its asci and spores. For this purpose the omnipresent *Erysiphe commune* on *Polygonum aviculare* is exceptionally favorable, because, after the material has been fixed and has been brought into alcohol, the whole mycelium, with the developing perithecia, may be stripped from the leaf without the slightest difficulty, thus avoiding the necessity of cutting the leaf in order to get the fungus. The stage in which the perithecia are still white or yellowish is the most favorable for sections. At this stage the material, when abundant, can be stripped off from the leaves before fixing. Sections should not be thicker than 5μ. About 3μ is best for free nuclear stages in the ascus and for the development of the ascospores. The safranin, gentian-violet, orange combination seems to give the best reults, although cyanin and erythrosin are quite satisfactory when the stains are properly balanced.

The Xylariaceae.—Most of these forms, in their mature condition, are black. In younger stages the color is lighter, often showing gray, brick-red, or brownish tints. *Nummularia* is common on dead branches of beech, elm, oak, locust, and other trees. It is generally flat, orbicular, or elliptical in form. *Ustilina* is a crustaceous form, rather diffuse and irregular in shape. It is most common on the roots of rotten stumps. *Hypoxylon* is more or less globose in form, and the color is brick-red, brown, or black. It is found on dead twigs and bark of various trees, especially beech, and is more abundant in moist situations. *Xylaria* (Fig. 45) is found on decaying stumps and logs, and often apparently on the ground, but really growing on twigs, wood, and bark just under the surface. When mature it is black outside and white or light-colored within. When young, it is easily cut in paraffin; in some forms the ascospores are fully formed before the stroma becomes hard enough to occasion any

difficulty in cutting. When the stroma becomes black, *Xylaria* is very hard and brittle, so that sections are likely to be unsatisfactory. For general morphological study, it is better to break the stroma transversely and examine with the naked eye and with a pocket lens. The asci with their spores can be teased out and mounted in water. For permanent preparations, soak the stroma for a month in equal parts of 95 per cent. alcohol and glycerine; then cut sections, and after leaving them in glycerine for a day or two, mount in glycerine jelly. It is better not to stain the old stages. (Fig. 46.)

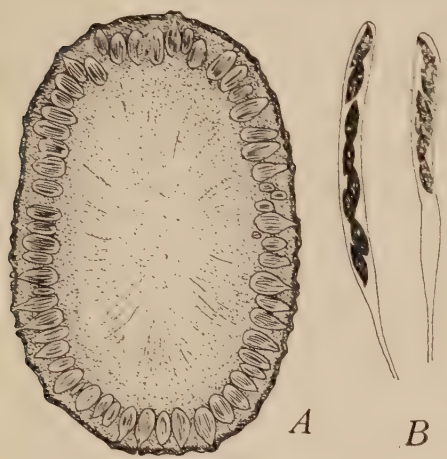

FIG. 46. · *Xylaria*

A, transverse section of a young stroma showing perithecia. × 8. Fixed in chromo-acetic acid, stained in bulk in alum carmine, imbedded in celloidin, and mounted in balsam. *B*, two asci with spores. × 245. The mature stroma was soaked for several days in equal parts of 95 per cent. alcohol and glycerine, and then imbedded in celloidin. Not stained.

Other forms of similar consistency may be treated in the same way.

THE LICHENS

The lichens are usually regarded as difficult forms. In younger stages they occasion no trouble, but an old apothecium or a leathery thallus often fails to cut well. Difficulties may be minimized by using prolonged periods. The following schedule has proved satisfactory for the thalli and mature apothecia of *Physcia*, *Usnea*, *Sticta*, *Collema*, *Parmelia*, and *Peltigera*:

1. Chromo-acetic acid (medium solution, p. 28), 2 to 4 days.
2. Wash in water, 6 to 24 hours.
3. 15, 35, 50, 70, 85, and 95 per cent. alcohols, 6 to 24 hours each.
4. 100 per cent. alcohol, 1 to 2 days, changing 2 or 3 times.
5. Mixtures of alcohol and xylol, 1 to 2 days.
6. Pure xylol, 6 to 24 hours.
7. Xylol and paraffin on the bath, 5 to 6 days.
8. Paraffin at 54° to 60°, changing once or twice, 6 to 10 hours.
9. Imbed in as thin cakes as possible.

Cyanin and erythrosin is a very good stain for lichens. The algæ stain blue and the filaments of the fungus take the red. Where the association of the alga and the fungus is rather loose, as in *Dichonema*, more satisfactory mounts can be made by staining in eosin, or hæm-alum and eosin, and then teasing slightly with needles and mounting in glycerine.

BASIDIOMYCETES

This is an immense group, of which the smuts, rusts, mushrooms, toadstools, puffballs, and bracket fungi are the most widely known representatives.

The Smuts (*Ustilagineae*).—The smuts are abundant on wheat, oats, corn, and various other plants.

The smuts may be studied in the living material. The following method, recently described by Ellis, is worth remembering: A supply of smutted barley may be obtained by sowing soaked, skinned barley that has been plentifully covered by *Ustilago* spores. In such material it is easy to trace stages in the development of spores. Free-hand sections of ears about three-eighths of an inch long show the mycelium and spore clusters. If smutted ears be removed and kept floating on the water, the spores continue to develop and often germinate. For paraffin sections desirable stages should be fixed in Flemming's fluid or picro-acetic acid. Delafield's hæmatoxylin, followed by a very light touch of erythrosin or acid fuchsin, will give a good stain.

For a study of the germinating spores and conidia, cultures may be made in beerwort on the slide or in watch crystals. Harper's method of making preparations from such material is ingenious and will undoubtedly prove valuable in making mounts of various small plant and animal forms. A drop of the material is taken up with a capillary tube and is then gently blown out into a drop of Flemming's weaker solution (15 minutes or an hour was sufficient for the fungus spores). Cover a slide with albumen fixative, as if for sections. A drop of the material, without previous washing, is drawn up into the capillary tube and touched lightly and quickly to the surface of the albumen. A series of such drops, almost as

small as the stippled dots in a drawing, may be applied to the slide. The fixing agent may now be allowed to evaporate somewhat, but the preparation must not be allowed to dry. As the slide is passed rapidly through the alcohols, the albumen is coagulated, and the preparation may be treated just as if one were dealing with ribbons of sections.

The Rusts (*Uredineae*).—*Puccinia graminis*, the common rust of wheat and oats, is familiar to everyone. The uredospores, or summer spores, known as the red rust, and winter spores, known as the black rust, are found in unfortunate abundance, but the æcidium stage on the barberry is not necessary for the vigorous development of rust in the United States, and is seldom found. Most teachers are obliged to depend upon botanical supply companies for this material. There are, however, various æcidia which are as good, or even better, for morphological study. The æcidia growing on *Euphorbia maculata* (spotted spurge), and on *Arisaema triphyllum* (Jack-in-the-pulpit) are much easier to cut, and seem easier to stain. Delafield's hæmatoxylin, followed by a very light stain in erythrosin, is good for both æcidia and spermagonia, especially after Flemming's fluid. It is rather difficult to get good sections of uredospores and teleutospores, because the leaves of wheat and oats are refractory objects to cut. The cutting is easier after picro-acetic acid than after corrosive sublimate or the chromic-acid series. (Fig. 47.)

Everyone who studies the rusts should attempt to germinate the uredospores and teleutospores. For this purpose the hanging-drop culture may be employed, as described in the chapter on temporary mounts. The uredospores germinate readily all summer, but it is said that the teleutospores will germinate only in the spring following their maturity. However, the teleutospores of many species, like *Puccinia xanthii* on *Xanthium canadense* (cocklebur), will germinate as soon as they ripen, and will serve equally well for study. If a particularly good specimen is secured, it may be preserved by the method previously described for desmids, except that in this case it might be worth while to attempt staining with Mayer's hæm-alum, or with eosin.

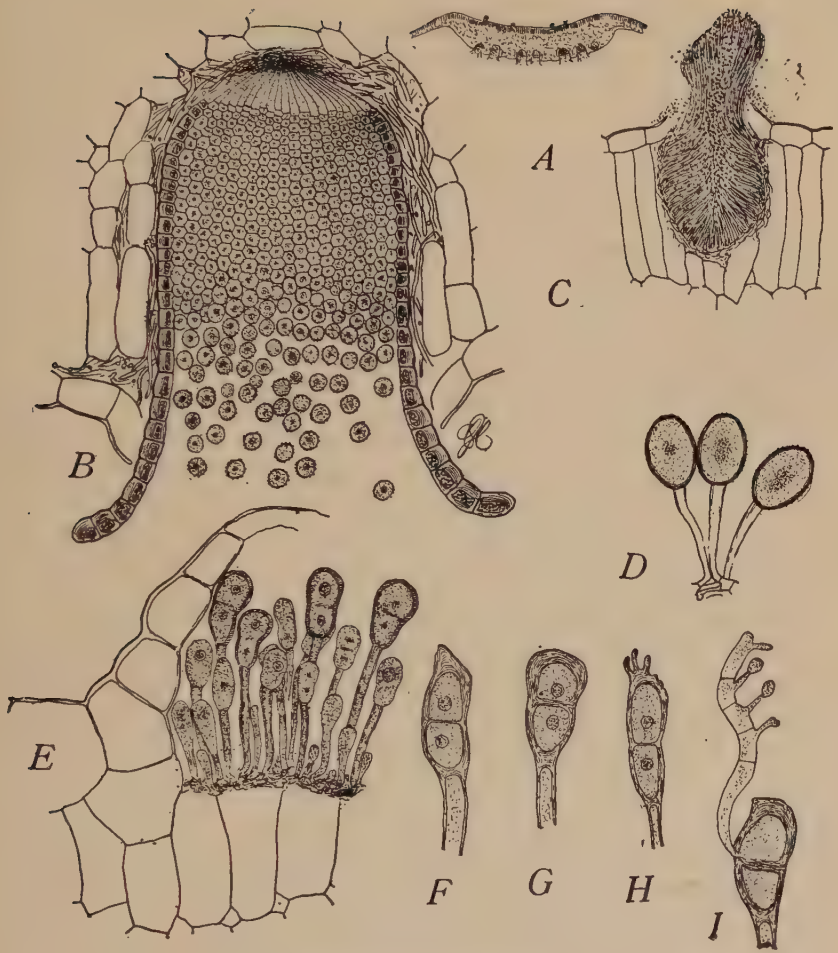

FIG. 47.—*Puccinia graminis*

A, transverse section of barberry leaf showing æcidia and spermagonia. × 7. *B*, longitudinal section of a single æcidium. × 192. Fixed in Flemming's weaker solution and stained in Delafield's hæmatoxylin. *C*, a single spermagonium. × 192. Fixed and stained as in *B*. *D*, three uredospores growing on oats. × 375. Fixed in 2 per cent. formalin, stained in bulk in alum carmine, and teased out in glycerine. *E*, section of young teleutospores on oats. × 375. Fixed in picro-acetic acid and stained in cyanin and erythrosin. *G*, *F*, *H*, three ripe teleutospores from a leaf of oats showing variation in form. × 375. *I*, germinating teleutospores. × 375.

The Fleshy Fungi.—All the fleshy fungi fix well in chromo-acetic acid, with 1 g. of chromic acid and 2 c.c. of glacial acetic acid to 200 c.c. of water. From 10–24 hours is sufficient. Wash in water, 6–24 hours, and imbed in paraffin in the usual way.

In the common shaggy mane mushroom, *Coprinus comatus* (Fig. 48), or in any form in which the spores change color with age, it is easy to select stages in the field. Where the gills are reddish or brownish at the edge, but lighter colored farther back, cut out quarter-inch cubes showing the whole range of color. Sections perpendicular to the surface of the gills and at right angles to the surface of the pileus, will show a fine series of stages in the development of spores. Some of the smaller species of mushrooms which allow the entire pileus to be cut transversely, make beautiful preparation. Sections showing the spores on the basidia should be about 10–15 μ in thickness, but to show the internal structure of spores and the nuclear phenomena in the basidia, sections must not be thicker than 4 or 5 μ. Cyanin and erythrosin is a good stain for general views, but the safranin, gentian-violet, orange combination is better for nuclei. Magdala red and anilin blue is also a good combination for the nuclei.

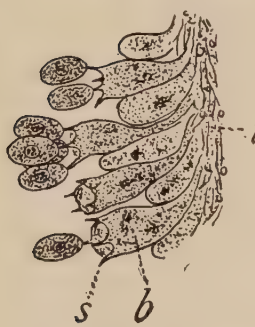

FIG. 48.— *Coprinus comatus*

Transverse section of a portion of one of the gills showing a part of the trama, *t*, and several basidia, *b*, each with four sterigmata, *s*. Spores have fallen off from many of the sterigmata. × 750.

In *Hydnum* and *Polyporus*, cut out pieces about three or four spines or three or four pores in width and about half an inch long. A rectangular piece which will allow the transverse sections of the spines or pores to be about one-eighth of an inch wide and half an inch long, cuts better than a piece which will give square sections.

In *Boletus*, simply strip off the hymenium and cut into pieces which will give transverse sections of the tubes.

In *Lycoperdon*, *Bovista*, *Geaster*, and *Scleroderma*, longitudinal sections of the entire fructification can be cut in paraffin, as long as these forms are less than an inch in diameter.

Young stages of *Cyathus*, *Crucibulum*, and *Nidularia* cut easily in paraffin; somewhat older stages can be cut in celloidin, but mature stages fail to cut by any of our present methods.

Very soft, watery forms, like *Tremella*, cut well in paraffin, but they should not be allowed to remain long in the paraffin bath. About 15–20 minutes is sufficient.

CHAPTER XVI

BRYOPHYTES

The Bryophytes, comprising the two groups of Liverworts (*Hepaticae*) and Mosses (*Musci*), present a great diversity of structure, some being so delicate that good preparations are very uncertain, while others are so hard that it is difficult to get satisfactory sections. Between these extremes however, there are many forms which readily yield beautiful and instructive preparations.

If but one fixing agent should be suggested for the entire group, it would be chromo-acetic acid with 1 g. chromic acid and 2 c.c. acetic acid to 200 c.c. of water. It should be allowed to act for about 24 hours. Many delicate forms may be mounted whole in Venetian turpentine, glycerine, or glycerine jelly. Where sections are needed, cut them in paraffin, if possible, using celloidin only as a last resort for refractory structures which resist infiltration. As one gains in experience and carefulness, the number of cases which seem to demand celloidin will become fewer and fewer.

Instead of treating forms in a taxonomic sequence, we shall consider first the gametophyte structures under the headings *thallus*, *antheridia*, and *archegonia*, and shall then turn our attention to the *sporophyte*.

HEPATICAE

Some of the liverworts are floating aquatics, but most of them grow on logs or rocks or upon damp ground. They are found at their best in damp, shady places. Many of them may be kept indefinitely in the greenhouse. *Riccia*, *Ricciocarpus*, *Marchantia*, *Conocephalus*, *Asterella*, and many others vegetate luxuriously, and often fruit if kept on moist soil in a shady part of the greenhouse, and they do fairly well in the ordinary laboratory if covered with glass and protected from too intense light.

Marchantia, and similar forms are not difficult to establish out of doors. A rather damp, shady spot close to the north side of a building is best. Scrapings from a board which has been nearly burned up makes the best fertilizer to scatter on the soil, if one is to cultivate *Marchantia*. Such freezing as *Marchantia* receives in the vicinity of Chicago does not prevent it from appearing again the next spring. If it is desirable to have material throughout the year, the out-of-door culture may be made in a box which can be brought into the laboratory or greenhouse in the winter. A box three feet long, two feet wide, and one foot deep will be convenient. It should have a glass cover; an old window will do. There should be about six inches of dirt in the box. A mixture of sand, loam, and charred scrapings will make a good substratum for *Marchantia*. If one is to raise liverworts in the laboratory, it is absolutely necessary to note carefully the conditions under which they grow in the field.

The living plants are very desirable, since they not only furnish the best possible material for habit work and the coarser microscopic study, but they also enable one to secure complete series in the development of the various organs.

The Thallus.—In many cases it will not be necessary to make a special preparation for the study of the thallus, since preparations of antheridia, archegonia, or sporophytes may include good sections of vegetative portions. This is particularly true of forms like *Riccia*, where the various organs are not raised above the thallus. In forms like *Marchantia*, where the antheridia, archegonia, and sporophytes are borne upon stalked receptacles, it is better to make separate preparations to show the structure of the mature thallus. Sections intended to show the structure of the mature thallus should be 15 μ to 25 μ in

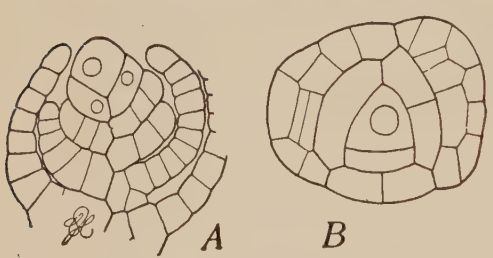

FIG. 49.—*Ptilidium ciliare*. × 420

A, longitudinal. *B*, transverse section of the leafy gametophyte. Fixed in Flemming's weaker solution, stained in a mixture of acid fuchsin and iodine green. Ten microns.

thickness, but sections to show the growing point and development of the thallus should not be thicker than 10 μ. Material showing apical cells and development of the thallus is easily got into paraffin, even in forms like *Ricciocarpus*, which in their mature condition are in danger of collapsing. The apical region of the *Jungermanniaceae* (Figs. 49 and 50) affords an excellent opportunity for studying the development of the plant body from a single apical cell.

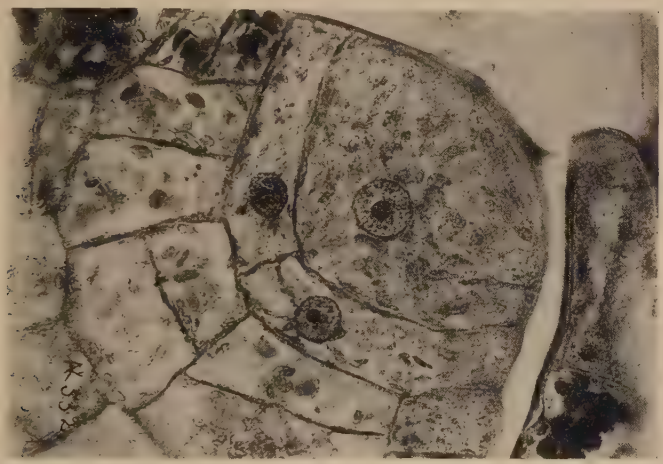

FIG. 50.—*Pellia epiphylla*, photomicrograph of apex of gametophyte showing apical cell and segments. Safranin, gentian-violet, orange. The negative was made by Dr. Köhler, of the Zeiss factory, in Jena, Germany.

If mixtures containing osmic acid are used for fixing, there may be a difficulty in the staining, even after using peroxide of hydrogen.

Chromo-acetic mixtures, without osmic acid, are better for the apical region. Chromo-acetic acid, followed by Delafield's hæmatoxylin, is good for the apical cells and developing regions, but a light counter-stain with erythrosin improves preparations of the mature thallus. In forms like *Pellia*, where even the apical cells are more or less vacuolated, a sharp stain in safranin and gentian-violet is quite satisfactory, bringing out not only the cell walls, but also the various cell contents (Fig. 50). The chloroplasts and leucoplasts are well differentiated by this stain. After corrosive sublimate-acetic, a vigorous staining in a mixture of acid fuchsin

and iodine green often brings out the walls very sharply. After corrosive sublimate-acetic the material may be stained in bulk with alum cochineal or alum carmine, thus giving fairly good preparations and saving considerable labor.

Antheridia.—If you have the material, it is not difficult to get good preparations showing the development of antheridia. In forms like *Asterella*, *Pellia*, etc., cut out a small portion of the thallus bearing the antheridia. The piece should not be more than a quarter of an inch square, and if it can be smaller, so much the better. For early stages of the antheridia of *Marchantia* select young antheridiophores which still lie close to the thallus. These readily cut as thin as 5μ, and a single slide will usually show a more complete series than is represented in the figure of *Asterella* (Fig. 51), but after the stalk begins to lengthen, the young stages become infrequent, and it is not always easy to cut thin sections. Delafield's hæmatoxylin or iron-hæmatoxylin serve very well for such stages as are shown in the figure. The protoplasm of the young antheridia is so dense that the addition of a counter-stain is almost sure to injure the preparation by obscuring the cell walls. For stages older than that represented in *D*, showing the development of the spermatozoid, the paraffin must be rather hard (melting at 55° C. to 58° C.), and the sections should not be thicker than 5μ, while 2μ or 3μ is thick enough. For such stages use the safranin, gentian-violet, orange combination, Haidenhain's iron alum-hæmatoxylin with or without a faint trace of erythrosin or orange G, or use a mixture of acid fuchsin and methyl green.

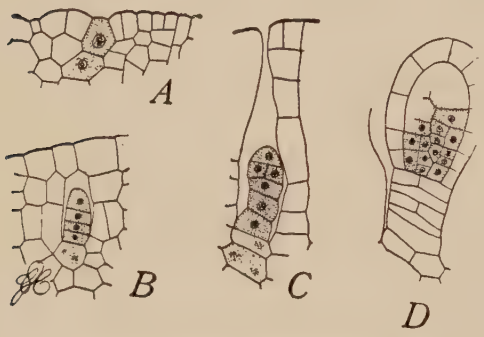

FIG. 51.—*Asterella hemisphaerica*. × 255

Successive stages in the development of antheridia. Fixed in chromo-acetic, stained with Delafield's hæmatoxylin. Section 10 microns thick.

Ikeno, in his work upon spermatogenesis in *Marchantia polymorpha*, used Flemming's solution diluted with the same

volume of distilled water, and strained in safranin, gentian-violet, orange.

If antherozoids are found escaping, transfer them to a small drop of water on a clean slide, invert the drop over a 1 per cent. solution of osmic acid for two or three minutes, allow the drop to dry up, pass the slide through the flame two or three times, as in mounting bacteria, and then stain sharply in acid fuchsin. This should show the general form of the antherozoid, and will usually bring out the cilia.

The Archegonia.—The methods for archegonia are practically the same as for antheridia. Too much stress cannot be laid upon the importance of carefully selecting the material. Use very small pieces, and, before placing them in the fixing agent, trim them to such a shape that the position of the archegonia will be known accurately even after the pieces are imbedded in paraffin. For stages like Fig. 52, *A* and *B*, Delafield's hæmatoxylin is a good stain, and 10 μ is about the right thickness. For stages like *C*, in such forms as *Marchantia*, where the necks are long and often somewhat curved, it is better for general purposes to use sections from 15 μ to 20 μ in thickness. If it is desired to obtain preparations showing the cutting off of the ventral canal cell, the development of the oösphere, and the process of fertilization, the sections should be from 5 μ to 10 μ in thickness, and the same staining may be used for the development of antherozoids. For archegonia containing young

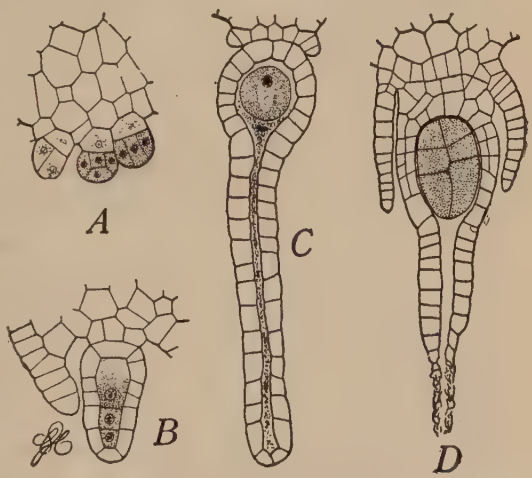

FIG. 52.—*Marchantia polymorpha.* × 400

A, three early stages in the development of the archegonia. Delafield's hæmatoxylin. *B*, young archegonium showing two-neck canal cells and the central cell before the cutting off of the ventral canal cell. Fuchsin and methyl green. *C*, mature archegonium just ready for fertilization. Safranin, gentian-violet, orange. *D*, young embryo. Delafield's hæmatoxylin.

embryos, like that shown in *D*, Delafield's hæmatoxylin without any counter-stain gives beautiful preparations when the staining is well done. It is easier for the beginner to get good preparations with the safranin, gentian-violet, orange combination.

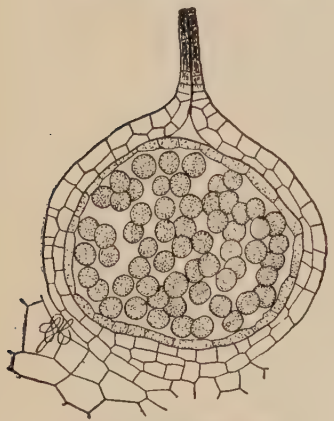

FIG. 53.—*Ricciocarpus natans.* × 104

Young sporophyte inclosed in the archegonium. Spore mother-cell stage. All the cells of the sporophyte except a single peripheral layer (dotted in the figure) produce spores. Fixed in picro-acetic acid and stained in Delafield's hæmatoxylin. Celloidin section 30 microns in thickness.

In *Ricciocarpus*, unless the direction of the axis of the archegonium at every stage in the development be known, there will be very few median longitudinal sections.

In forms like *Porella* and *Scapania*, the involucre is likely to hold a bubble of air which will delay or even prevent fixing. The best plan is to cut off the offending leaf with a pair of slender pointed scissors. Sometimes the air can be got out with an air-pump. While heating, the fixing fluid will often drive out the air, the heating at this time exaggerates kinoplasmic activities and so gives unreal views in the finished preparation.

The Sporophyte.—Sporophytes in early stages of development often yield good preparations without very much trouble, but in later stages they are frequently difficult to cut on account of the secondary thickening of the capsule wall and the stubborn extine of the mature spores. It is hard to get *Ricciocarpus* into paraffin without shrinking, and the same thing may be said of other forms which have such loose tissue with large air cavities. Formerly, we resorted to celloidin for stages like that shown in Fig. 53.

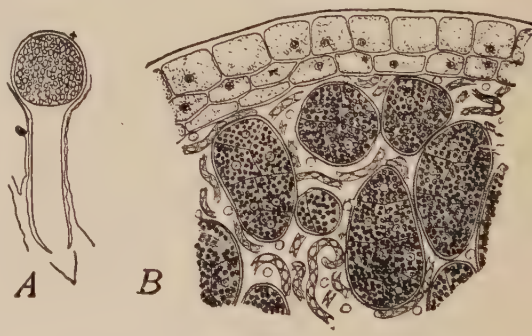

FIG. 54.—*Pellia epiphylla*

A, habit sketch of sporophyte. × 10. *B*, small portion of sporophyte (at × of *A*), showing the capsule wall, the spores, and the elaters. Fixed in chromo-acetic acid and stained in cyanin and erythrosin. Ten microns.

The difficulty here is occasioned by the hardened neck of the archegonium. This difficulty and also the danger of shrinking due to the large air cavities may be overcome by fixing for 24–36 hours in chromo-acetic acid and keeping the material for a week in the xylol and paraffin mixture. Not more than 2 or 3 hours in the bath will be needed to complete the infiltration.

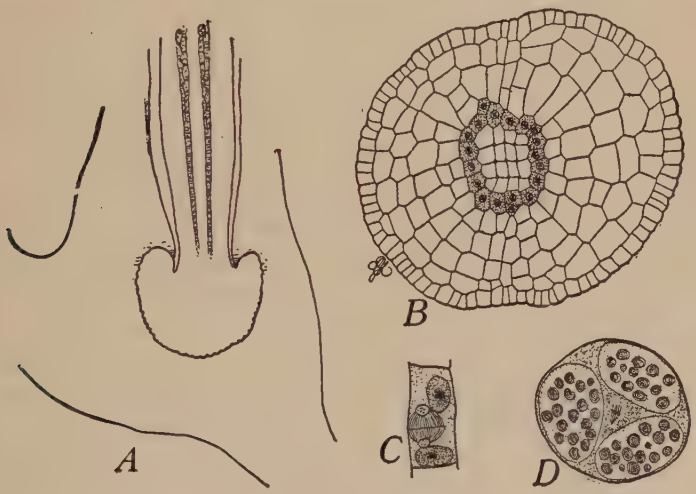

FIG. 55.—*Anthoceros laevis*

A, longitudinal section of lower portion of sporophyte imbedded in the gametophyte. × 45. *B*, transverse section of lower portion of sporophyte. × 200. Delafield's hæmatoxylin. Ten microns. *C*, vegetative cell from lower portion of the sporophyte. × 560. Fixed in Flemming's weaker solution and stained in a mixture of acid fuchsin and iodine green. Five microns. *D*, spore mother-cell showing three of the four chloroplasts with numerous starch grains. The nucleus is in the metaphase of the first division. × 560. Fixed in Flemming's weaker solution, stained in safranin, gentian-violet, orange. Five microns.

Forms like *Pellia* cut well in paraffin, especially in younger stages, but even in case of mature sporophytes it is not necessary to resort to celloidin. In *Pellia* and *Conocephalus* the spores are very large and have a rather thin wall. Both these genera show a peculiar, intrasporal development of the gametophyte, i. e., the gametophyte develops to a considerable extent before it ruptures the spore wall. (Fig. 54.)

For such stages and for later stages, the calyptra should be removed, and a small slab should be cut from each side of the

capsule. Mitotic figures during the first three divisions in these spores are exceptionally beautiful and are very easy to stain with the safranin, gentian-violet, orange combination, the chromosomes taking a very brilliant red, while the asters take the violet. Centrospheres are quite prominent during these three divisions. For the older sporophytes of *Marchantia* it is better not to cut the whole receptacle, but rather to remove the branches so that they may be cut separately. For the very best preparations of mature sporophytes it will pay to trim away the gametophyte structures, leaving only enough to show the foot with a few of the surrounding cells. Sections 5 μ to 10 μ thick can be made without much difficulty from material prepared in this way.

Among the Bryophytes no form affords a better opportunity for studying the development of spores than *Anthoceros*, since a single longitudinal section of the sporophyte may show all stages, from earliest archesporium to mature spores (Fig. 55). For studies like *A* and *B*, chromo-acetic material cut to 10μ thick and stained in Delafield's hæmatoxylin is very good. The starch grains in the chloroplasts take a beautiful violet color with the safranin, gentian-violet, orange combination. With so many stages in a single section, it will be impossible to stain all of them well. A stain which will show the mother-cells and their divisions will be too deep for the mature spores, and a stain which shows the spores well will be too faint for the mother-cells. It is very difficult, to bring out the details of nucleus or chloroplast on account of the minute size of these structures. The drawings from which *C* and *D* were reproduced were made with a one-sixteenth oil immersion objective. The drawings, like all the others illustrating the Bryophytes, were reduced one-half by photography.

CHAPTER XVII

BRYOPHYTES

MUSCI

Material for a study of the mosses is much more abundant, and a series of stages in the development of the various organs is easily secured; but it is much more difficult to obtain good preparations, because so many of the structures are hard to cut. Chromo-acetic acid is to be recommended as the most satisfactory fixing agent, but where structures are refractory and very likely to make trouble in cutting, it will often be found more satisfactory to use picro-acetic acid in the 70 per cent. alcohol, since material fixed in this regeant does not become as hard or as brittle as that fixed in any of the chromic-acid series.

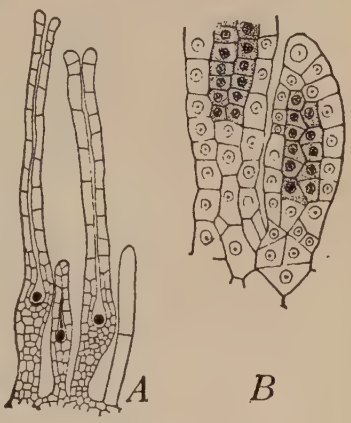

FIG. 56

A, archegonia of *Webera candicans*. × 104. Celloidin section. Twenty microns. *B*, young antheridia of Polytrichum commune. × 420.

Protonema.—Protonema of some moss can always be found at any season. Look for greenish patches resembling *Vaucheria*. Such mats show the developing protonema and young leafy plants. Very young mats of moss will also show good protonema, but are not likely to show young buds. The brownish bulbils, which are quite common in mosses, can be seen with a good pocket lens. The little *Webera*, almost always found on the pots in the fernery or on the benches in greenhouses, quite frequently shows this mode of reproduction. Protonema is easily grown from spores.

Permanent mounts are very easily made. Simply wash away the dirt with water and put the material into 10 per cent. glycerine, and let the glycerine concentrate. Mount in glycerine or

glycerine jelly. Such preparations, made seven years ago, still show the characteristic green and brown colors.

Antheridia.—It is easy to find material for a study of antheridia, because, in so many cases, the antheridial plants can be detected at once without even a pocket lens. *Funaria*, with its bunch of antheridia as large as a pinhead, is extremely common everywhere. Spring is the best time to collect it, but it is found fruiting in the autumn and sometimes in summer; besides, it is easily kept in the greenhouse, where it may fruit at any time. *Bryum proliferum* has a still larger cluster of antheridia, which may be seen at a distance of several yards. *Polytrichum* also has a large cluster of antheridia surrounded by reddish leaves, so that the whole is sometimes called the moss "flower." In making preparations of *Polytrichum* these colored leaves should be carefully removed after the material has been got into 70 per cent. alcohol. A single antheridial plant of *Polytrichum* often furnishes a fairly complete series of stages in the development of antheridia. (Fig. 56, *B*.) In all cases the stem should be cut off close up to the antheridia, for many of the moss stems cut like wire. It is not necessary to use celloidin for antheridia, nor is it desirable,

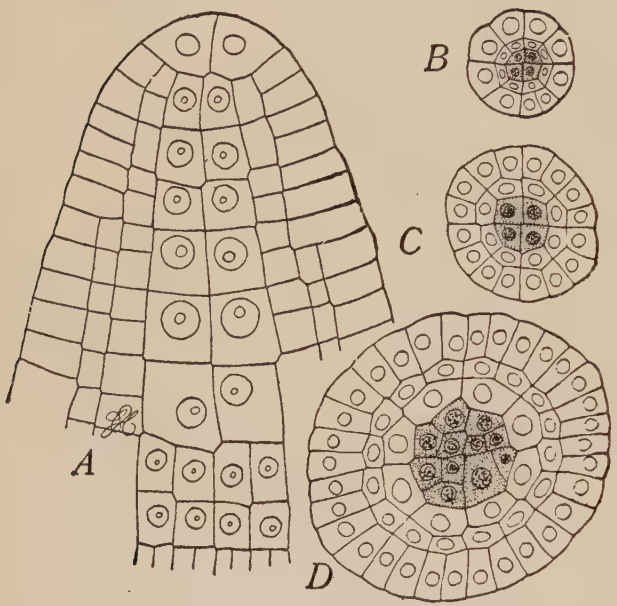

FIG. 57.—*Funaria hygrometrica*

A, apex of young sporophyte showing endothecium and amphithecium. × 420. Chromo-acetic acid and Delafield's hæmatoxylin. Ten microns. *B*, *C*, and *D*, transverse sections of a sporophyte of the same age as *A*, taken at three different levels. × 255. Ten microns.

except where sections from 20 μ to 50 μ thick are wanted for habit work. Delafield's hæmatoxylin is recommended for staining.

It is far better to study such stages in the fresh material. When a particularly fine view is secured in this way, a permanent preparation may be made by putting the piece into 10 per cent.

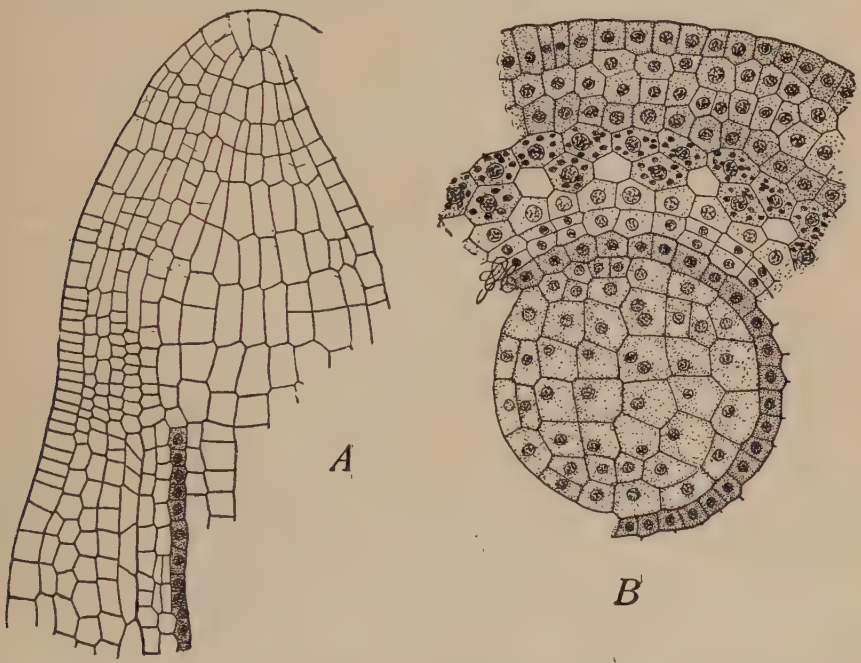

FIG. 58.—*Funaria hygrometrica.* × 420

A, longitudinal section of capsule. *B*, transverse section of capsule of about the same age as *A*. The columella, archesporium, outer spore case, two layers of chlorophyll-bearing cells, and the beginning of the air spaces can be distinguished at this stage. Delafield's hæmatoxylin and erythrosin. Ten microns.

glycerine, without any fixing or staining, and allowing the glycerine to concentrate. Then mount in glycerine jelly.

Archegonia.—Since the necks of the archegonia are usually long and more or less curved, it is necessary, for habit work, to cut sections as thick as 20 μ or 30 μ in order to get a view of an archegonium in a single section. Celloidin may be used for such preparations; but here, as in the case of the antheridia, it is better to use fresh material, and to depend upon glycerine or glycerine

jelly, for permanent mounts. For the development of the archegonium, the oösphere, the canal cells, and also for the process of fertilization, it is better to use paraffin. (Fig. 56, *A*.)

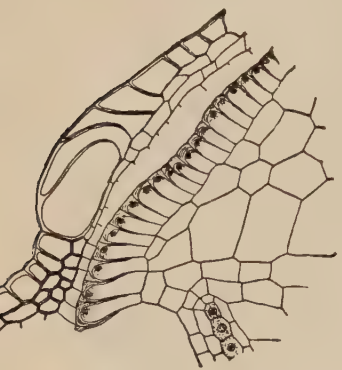

FIG. 59.—*Bryum*. × 200

Portion of a nearly mature capsule showing operculum, annulus, peristome, and three cells of the sporogenous tissue. Fixed in Flemming's weaker solution, stained in safranin and Delafield's hæmatoxylin. Fifteen microns.

Sporophyte.—It is often difficult to get good mounts of sporophytes. In the younger stages the calyptras are likely to interfere with cutting, while in the older stages the peristome, or hard wall of the capsule, occasions the trouble. If an attempt is made to remove the calyptra in young stages, like *A* of Fig. 57, the apex of the sporophyte usually comes with it. While picro-acetic acid material cuts more easily, chromo-acetic acid followed by Delafield's hæmatoxylin gives so much sharper differentiation in stages like those shown in Fig. 58 that it is better to use harder paraffin (55° to 58° C.) and make an effort to get preparations from chromic material.

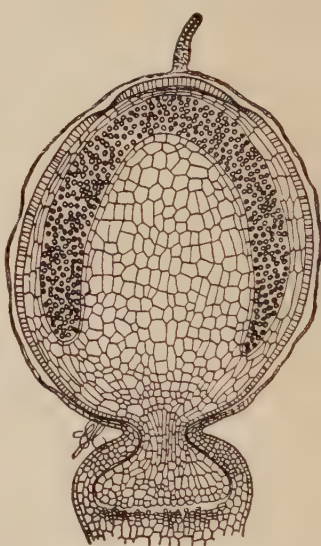

FIG. 60.—*Sphagnum*. × 24

Longitudinal section of mature sporophyte, showing also the upper portion of the pseudopodium and the calyptra. Chromo-acetic acid, Delafield's hæmatoxylin. Paraffin. Ten microns.

Stages like that shown in Fig. 58 are cut with comparative ease, for the calyptra is easily removed, and the capsule wall is not yet hard enough to occasion any difficulty. The cell walls are so easily stained in moss capsules that a light counter-stain with erythrosin or acid fuchsin may be used to bring out the cytoplasm and plastids without appreciably obscuring the cell walls. *Funaria* and *Bryum* afford an excellent study in the development of the capsule, since all the structures of a highly differentiated moss sporophyte are present, and *Bryum* is particularly easy to cut in stages like those shown in Fig. 59.

Sporophytes, in their more mature stages, are almost sure to present considerable difficulty in cutting. For general work fairly good preparations may be gotten from celloidin material, but it is worth while to try paraffin, for it is sometimes successful, and when it does succeed it is far superior. As soon as the cell walls begin to thicken, as in the development of the peristome, safranin is an excellent stain, and this, followed by Delafield's hæmatoxylin, will give an elegant differentiation in the older stages of the sporophyte. After capsules have begun to turn brown it will be almost impossible to infiltrate them unless they are pricked with a needle.

The mature sporophytes of *Sphagnum* (Fig. 60) are exceptionally hard to cut. It will be worth while to prick the capsule with a needle when the material is collected. This will allow the fixing agent to penetrate readily, and will also facilitate the infiltration of paraffin or celloidin. The puncture causes only a slight damage, and need not reach the really valuable portion which is to furnish the median longitudinal sections.

The younger stages in the sporophyte of *Sphagnum*, and also the antheridia, archegonia, and the peculiar development of the leaves are easily cut in paraffin.

CHAPTER XVIII

PTERIDOPHYTES

This group, including the Filicales, Equisetales, and Lycopodiales, or, more popularly, the ferns, horsetail rushes, and club mosses, is familiar to everyone. Material is abundant, and so easily recognized that anyone who pays a little attention to collecting can, in a single season, get a fine supply for a study of the group. Some desirable forms may not be present in all localities, but these will be few, and can be obtained at a reasonable price from those who make a business of collecting.

FILICALES

Without attempting to follow any taxonomic sequence, the methods of preparing the various structures of the homosporous forms will be presented, and then the peculiarities of the heterosporous members will be considered.

The Prothallia.— Prothallia can usually be found on the pots in the ferneries of greenhouses. Ripe spores of some fern or other can be obtained at any greenhouse at any time in the year, and spores of most of our native ferns germinate well and produce good prothallia, even if the sowing is not made for several months after the spores have been gathered.

Fine prothallia of *Pteris aquilina* have been grown two years after the spores were gathered. Some, however, must be sown at once, or they will not germinate at all. Spores which are large and contain enough chlorophyll to make them appear greenish, should be sown at once. The spores of the common *Osmunda regalis*, and of the other members of the genus, must be sown as soon as ripe, or they fail to germinate. The prothallia of *Osmunda regalis*, if carefully covered with glass, may be kept for a long time, and they become quite large. Prothallia of this fern in the writer's laboratory produced ribbon-like outgrowths

three-sixteenths of an inch wide, and often more than two inches in length. These prothallia continued to produce archegonia, antheridia, and ribbon-like outgrowths for more than a year, when they suddenly "damped off." Lang watered prothallia with a weak solution of permanganate potash, which kills the fungi, but does not injure the prothallia. He does not state the strength of the solution.

The prothallia of most ferns will grow for a long time under such conditions. *Pteris aquilina* and many other ferns often furnish a good supply of antheridia in three weeks after sowing, and the archegonia appear soon after, but it is well to make sowings six weeks before material is needed for use. In *Pteris aquilina* and in many others, if the spores are sown too thickly, only antheridial plants will be obtained. If prothallia are to produce archegonia, they must have sufficient room and nutrition. If there are no greenhouse facilities and the prothallia must be grown in the laboratory, it is a good plan to take a glass dish, ten or twelve inches in diameter and about two inches deep, put a layer of broken pieces of flower pots on the bottom, cover this with a layer of rich loam, and over this sprinkle a layer of fine, clean sand, since sand is much more easily washed away from the rhizoids than is the loam. The whole should now be thoroughly wet, but not so as to have water standing on the bottom. Sow the spores and cover with a tightly fitting pane of ground glass. There should be no need for moistening the culture again, for prothallia can be kept fresh and vigorous for several months, or even for a year, without any wetting. When it is desired to secure fertilized material, sprinkle the prothallia with water, and the young sporophytes will soon appear.

If prothallia are to be cut in paraffin, rotten wood is a much better substratum than any soil. The prothallia grow just as well, and the rotten wood cuts so easily that it is not necessary to remove it from the rhizoids. Damp pieces as large as one's fist may be placed under a bell jar, where a damp atmosphere can be maintained.

If greenhouse facilities are available, any gardener can grow

prothallia in abundance without any directions from those who want the material.

Fern prothallia of the usual type are excellent objects for testing fixing agents, since the prothallia, while still in the fixing agent, may be examined with the microscope, and fluids which cause plasmolysis may be rejected. It will sometimes happen that plasmolysis may be avoided by varying the proportions of the ingredients of a fixing agent. Chromo-acetic acid with about 0.6 g. chromic acid and 0.4 c.c. of acetic acid to 100 c.c. of water will seldom cause plasmolysis, and will usually insure good fixing. It is a mistake to suppose that because prothallia are such delicate subjects the fixing will take but a few minutes. Chromo-acetic acid should be allowed to act for about 24 hours. Flemming's weaker solution will fix fern prothallia in 8–16 hours. If hot corrosive sublimate-acetic acid or hot picro-acetic be used, the fixing requires only 2 or 3 minutes, but results are not as uniformly successful as with members of the chromic-acid series.

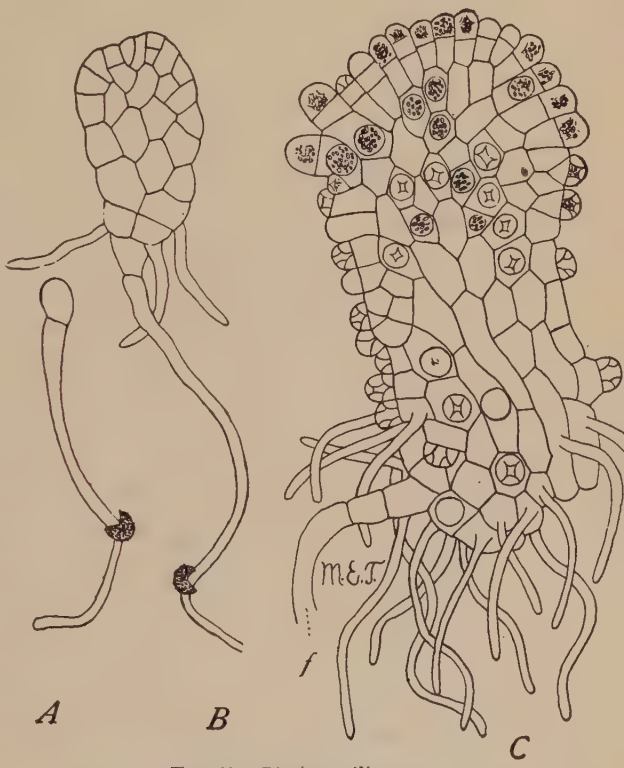

FIG. 61.— *Pteris aquilina*

A, filamentous stage. *B*, the apical cell has been established and several segments have been cut off. The figure shows the initial rhizoid, and also three rhizoids coming from the main body of the prothallium. *C*, an older prothallium covered with antheridia in various stages of development. From a glycerine mount, fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin. (From a drawing by Miss M. E. Tarrant.)

After any of the chromic-acid series, 2 or 3 hours' washing in water will be sufficient, if the water be changed as often as it becomes in the least degree discolored.

Of course, every student should study prothallia in the living condition. Permanent preparations of prothallia mounted whole (Fig. 61) should be used to show some features which were not so evident in the fresh mounts. Such preparations are most satisfactory in Venetian turpentine or in glycerine jelly. Fix in the chromo-acetic acid solution for 24 hours; wash in water 3–6 hours; and then proceed by one of the following methods:

a) Place the prothallia in 10 per cent. glycerine. After the glycerine concentrates, wash it out with 95 per cent. alcohol, stain in Magdala red and anilin blue, transfer to 10 per cent. Venetian turpentine, and proceed as usual.

b) Stain in Delafield's hæmatoxylin, and when the stain is satisfactory, transfer to 10 per cent. glycerine. When the glycerine has concentrated, mount in glycerine jelly.

c) Stain in Delafield's hæmatoxylin, transfer to 10 per cent. glycerine, and when the glycerine has concentrated, wash it out with 95 per cent. alcohol and transfer to 10 per cent. Venetian turpentine, which should concentrate in the usual way. The first and third methods are quite satisfactory.

For paraffin sections such as are shown in Figs. 62 and 63, the material should be passed through the alcohols, allowing about three or four hours for each grade. The mixtures of xylol and absolute alcohol should take about six hours, and as soon as the pure xylol has been added, the piece of paraffin may be added at the same time. Half an hour or an hour in the bath will give good results. It would be worth while to determine the best duration of the bath for such objects. Some workers claim that 10 or 15 minutes is amply sufficient, and that there is less danger from shrinking, while others think that several hours is better, and that two or three days will do no damage, if the fixing has been thorough and the temperature is not allowed to become higher than 50° C. If cedar oil be used instead of xylol, there is less danger of collapse previous to the bath, but the bath itself must

usually be more prolonged, since the cedar oil is not so easily got rid of as the xylol.

For morphological purposes sections about 10μ thick are better than thinner ones. Delafield's hæmatoxylin, with or with-

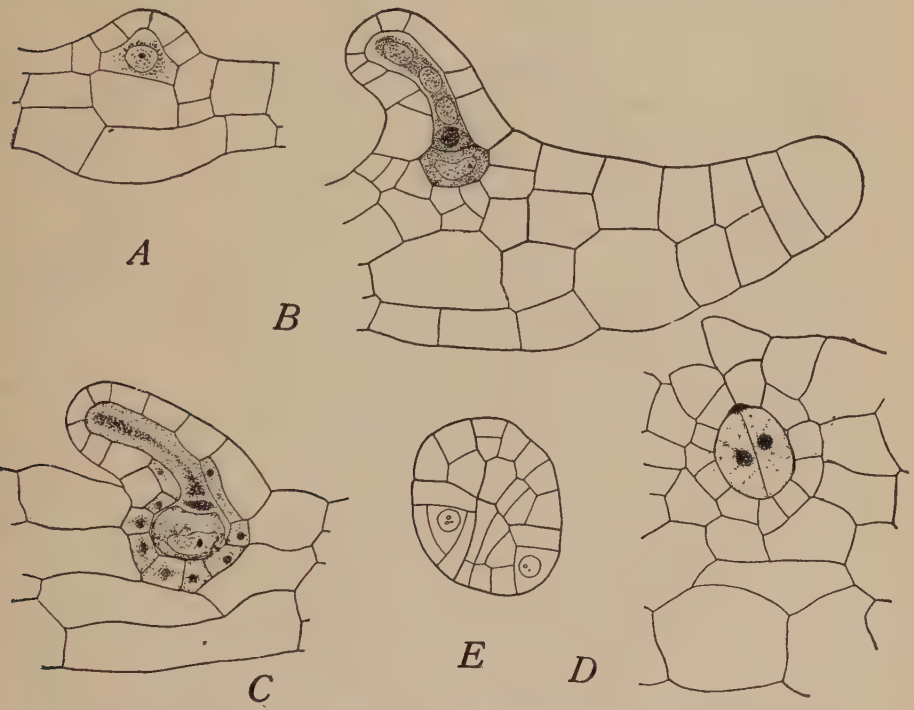

FIG. 62.—*Pteris cretica.* × 250

A, early stage in the development of the archegonium. *B*, later stage showing the oösphere, ventral canal cell, and *three* nuclei in the neck canal. *C*, still later stage almost ready for fertilization. The ventral and neck canal cells are breaking down, and the oösphere is nearly mature. Cells surrounding the oösphere have become richer in protoplasmic contents, and stain more deeply. *D*, first division of the embryo. *E*, young embryo still showing the outlines of the four quadrants. The apical cell in the lower left quadrant has cut off the first layer of the root cap. All drawn from material stained in bulk in alum carmine, a method not to be recommended.

out a counter-stain with erythrosin, is good for such sections as are represented in Fig. 62. If very thin sections are wanted for cytological study, it is better to use the safranin, gentian-violet, orange combination. For such views of antheridia as are shown in Fig. 63, Haidenhain's iron-hæmatoxylin seems to bring out the blepharoplast most sharply.

The peculiar tuberous prothallia of *Botrychium* and *Ophioglossum* are seldom found except by the experienced collector. The older prothallia, however, may be found by anyone who is able to recognize the young leaves of these plants when he sees them. Dig up young plants not more than three or four inches in height, and the prothallia, which persist for years, will often be found still attached. They are easy to cut and may be handled like other prothallia. Many attempts have been made to secure prothallia of *Botrychium* and *Ophioglossum* by sowing the spores, but no prothallia have been secured. As is well known, these prothallia are always infested by an endophytic fungus which seems to be a *Pythium*. It might be worth while to sow the spores in soil infected with *Pythium*.

FIG. 63.—*Pteris cretica*

A, section of a nearly mature antheridium showing the antherozoids inside. × 333 *B*, a developing antherozoid showing the blepharoplast drawn out into a deeply staining band. × 1900. Fixed in chromo-acetic acid and stained in Haidenhain's iron-hæmatoxylin.

The Sporophyte.—Methods for young sporophytes like those shown in Fig. 62, *D* and *E*, are the same as for archegonia and antheridia. The stage shown in *D* and also the quadrant and octant stages stain beautifully in the safranin, gentian-violet, orange combination, but for later stages Delafield's hæmatoxylin is decidedly better.

Root-tips to show the prominent apical cell are easily imbedded in paraffin. They should be cut 15μ to 20μ thick. Delafield's hæmatoxylin, without any contrast stain, is best for bringing out the prominent apical cell and its segments.

A leaflet with its sori, mounted whole, often makes an instructive preparation. Fix, wash, and stain lightly in eosin or erythrosin or do not stain at all, clear in xylol or clove oil, and mount in balsam. Mature sporangia mounted in this way give excellent views of the annulus.

Sections of the sporangia of any fern can be cut in paraffin.

Sporangia should also be cut in paraffin. *Pteris* is a good form for sporangia, since the long marginal sorus makes it

possible to get an immense number of median longitudinal sections of sporangia in a single preparation. (Fig. 64.)

Pteris cretica can always be found in fruit in greenhouses. Select a series of stages. The leaf should be cut with a razor—not with scissors—into pieces about one-fourth of an inch long. If sections of the whole leaf are not wanted only the marginal sorus need be cut off; in this way a much greater number of sporangia may be got on a slide. *Aspidium*, *Cyrtomium*, and *Asplenium* give beautiful views of the indusium covering the cluster of sporangia. The most satisfactory preparations will be got from material in which the sporangia have not yet begun to turn brown. Stages showing the spore mother-cell and its divisions to form the four spores stain best in the safranin, gentian-violet, orange combination.

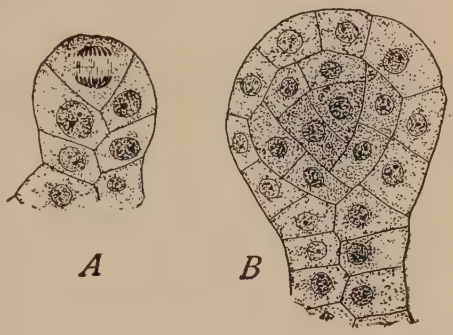

Fig. 64.—*Pteris cretica*. × 560

A, young stage in the development of the sporangium. *B*, older stage showing the tapetum. Fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin and erythrosin.

Botrychium furnishes excellent and usually accessible material for studying the development of sporangia of the eusporangiate type. For the archesporium and early stages in the development of the sporangia the material should be collected in the latter part of August or in September. The spore mother-cells in *B. virginianum* and *B. ternatum* are not formed until the following spring. When the sporangia for one year are ready to shed their spores, the sporangia for the next year will be found in early stages of development. When a frond is found, dig the plant up very carefully and remove the large frond. At its base will be found the frond for the next season, the sterile portion bent over so that its tip is directed downward and the fertile portion one-fourth to three-fourths of an inch in length—projecting from the ventral surface of the sterile frond. Cut off the fertile portion and fix it in a separate bottle, in order that it may be fixed rapidly, and also that it may not be injured by striking

against larger pieces, for it is worth while to preserve also portions of the stipes, rhizome, roots, and the rest of the bud. The bud contains, besides the large leaf which is to unfold the next year, two, three, or four leaf primordia which represent the leaves of the next two, three, or four years. Such a bud, cut off just below the insertion of the leaves, will furnish excellent longitudinal sections. The best views of the young sporangia are obtained from the fertile portion which has been removed. If the fertile portion has reached a length of half an inch or more, the individual pinnæ may be separated after the material is in xylol. This will make it easier to get good longitudinal sections of sporangia. Sections of this stage should not be thicker than 5μ. Iron-hæmatoxylin is the most satisfactory stain from the archesporium stage up to the mother-cell stage. For the mother-cell stage and later stages the safranin, gentian-violet, orange combination, or cyanin and erythrosin are better.

The rhizome, stipes, and root should be cut with a razor or very sharp knife, into pieces about one-fourth of an inch in length. The larger roots are better for transverse sections. The root-tips are unusually favorable for preparations of the apical cell. The upper part of the rhizome cuts easily, but all parts of this plant, even the older portions of the rhizome, can be cut in paraffin. Very useful preparations may be made by cutting transverse sections of the rhizome, stipe, and root, and then putting a piece of each of the three ribbons on each slide. The following schedule for paraffin sections will give elegant mounts of the root, stipe, and rhizome:

1. Stain in safranin, about 24 hours.
2. 50 per cent. alcohol until little or no safranin is left in the cellulose walls. Use a very weak acid alcohol, if necessary. If acid is used it must be washed out thoroughly before proceeding further.
3. Delafield's hæmatoxylin, 5 to 10 minutes.
4. Water, 15 minutes.
5. 50 per cent. alcohol, a few seconds.
6. Acid alcohol—as used for the safranin—a few seconds.
7. Wash in 50 per cent. alcohol until the purple color returns. The lignified walls should now show a brilliant red and the cellulose

walls a rich purple. If either stain is too deep or too faint, do not proceed any farther before making the necessary correction. If the safranin washes out, and the hæmatoxylin is too intense, shorten the period in the hæmatoxylin.

8. 95 per cent. alcohol, a few seconds.
9. 100 per cent. alcohol, 30 seconds to one minute.
10. Clove oil or xylol until cleared.
11. Balsam.

The spore mother-cells of *Osmunda* are excellent for a study of mitosis. The young sporangia of *O. cinnamomea* and *O. Claytoniana* show the mother-cell stage in the autumn, but the division into spores does not occur until the following spring, in the vicinity of Chicago the mitotic figures being found during the latter part of April. *O. regalis* does not reach the mother-cell stage in the autumn. Material for mitosis should be collected during the first two weeks in May. The material may be fixed in chromo-acetic acid or in Flemming's weaker solution. Sections should not be thicker than 10μ, and 5μ will be found satisfactory. With safranin and gentian-violet, it is particularly easy to stain the spindle violet and the chromosomes red.

Many stems and stipes cut easily, but sections of some of the larger and older stems and stipes seem to be impossible by the usual methods. It is practically impossible to get a thin, even section of a stem of *Alsophila* three or four inches in diameter. It is better to saw out a section about an inch thick, and then make the surface as smooth as possible. Such a section, when varnished, shows the topography very well. Near the growing point, where the tissues have not become hardened, sections two inches in diameter can be cut without any imbedding. Such sections, 20μ or 30μ in thickness make instructive preparations when mounted in balsam without any staining. Very large stipes present the same difficulties.

In *Osmunda*, and in many other ferns of similar habit, the rhizome is surrounded by the very hard leaf bases. Good sections of the central cylinder can be secured only by dissecting away these hard leaf bases and any hard portions of the cortex before attempting to cut sections. A short distance back of the

growing point will be found a region which will show practically all the structures of the mature stem, and which will be easy to cut. Even in this region the leaf bases should be dissected away. From the apical cell back to the region where the sclerenchyma is beginning to turn brown, the material is easily cut in paraffin. Older portions should be cut free hand or in celloidin.

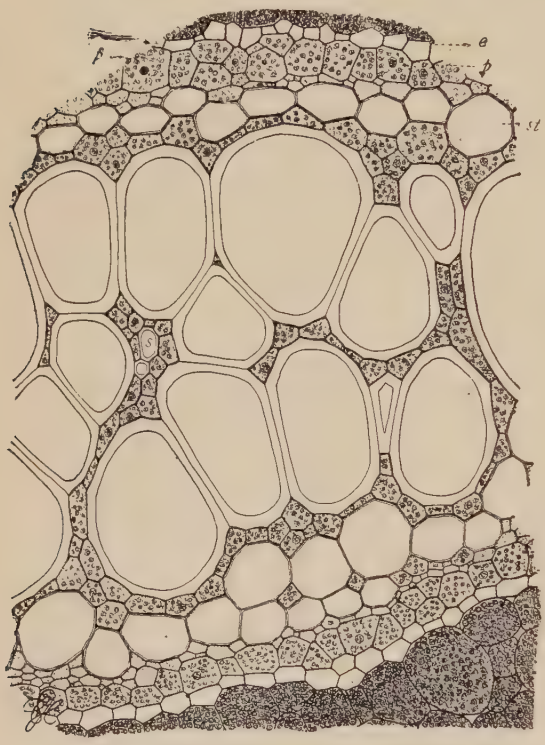

FIG. 65.—*Pteris aquilina*

A part of a transverse section of the vascular bundle of the rhizome. × 166. *e*, endodermis. *p*, pericycle. *st*, sieve tube. *t*, scalariform tracheid. Drawn from a celloidin section from material fixed in picro-acetic acid and stained in safranin and Delafield's hæmatoxylin.

The rhizomes of *Pteris aquilina* (Fig. 65) and similar forms of similar consistency may be cut free hand or in celloidin. In digging up rhizomes, do not merely dig down until the rhizome can be grasped and then pull it up, for such material is sure to show the pericycle of the bundles torn away from the parenchyma. Dig carefully around the rhizome and then cut off with a very sharp knife pieces about two inches in length. Put the fresh rhizome into the microtome and cut as thin sections as possible. Keep the knife wet with water and put the sections into chromo-acetic acid as soon as they are cut. After remaining in the acid 4–8 hours or over night, the sections are washed in water about an hour. They may then be stained in safranin and Delafield's hæmatoxylin or in methyl green and acid fuchsin, as directed in the chapter on "Free-Hand Sections."

Fairly good mounts may be made in much less time by putting the sections into 95 per cent. alcohol as soon as they are cut. Within 5 minutes they may be put into methyl green. The methyl green will stain fairly well in 1 hour. Wash until all or nearly all the methyl green is removed from cellulose walls, and then stain for about 1 minute in acid fuchsin. Dehydrate, clear in clove oil or xylol, and mount in balsam.

Celloidin sections may be stained in Delafield's hæmatoxylin and eosin without dissolving away the celloidin matrix. This keeps in place the cell contents which are likely to wash out if the matrix is removed. If safranin and Delafield's hæmatoxylin or methyl green and acid fuchsin are used, the celloidin matrix may be dissolved away either before staining, when it may be done with ether alcohol, or after staining, when it is better to remove it with clove oil.

FIG. 66.—*Marsilea quadrifolia.* × 333

A, apex of megaspore with archegonium containing the oösphere. Large starch grains are shown beneath the archegonium. *B*, young embryo. Both fixed in picro-acetic acid and stained in Delafield's hæmatoxylin. (From a drawing by Miss M. E. Tarrant.)

The Heterosporous Filicales.—*Marsilea* (Figs. 66 and 67) is the most available and convenient laboratory type of this group. *Marsilea* is easily grown in a pond or in an aquarium in the greenhouse. In setting it out in a pond, select a place with a gently sloping bank, so that part of the material may be under water and part may creep up the bank. In the greenhouse, a rectangular aquarium may be tilted to secure the same conditions. The portions which are not under water will continue to fruit during the summer and autumn. The whole sporocarp cuts easily in paraffin during the development of sporangia, the division of the spore mother-cells, and even during the earlier

stages in the formation of spores. Except in the case of the youngest sporocarps, it is better to cut off a small portion at the top and at the bottom to facilitate fixing and infiltration. The mother-cell stage and the young spores will be found in sporocarps which are just beginning to turn brown. In nature, no further nuclear divisions take place within the sporangium until the next spring, but the wall of the sporocarp becomes extremely hard. Sporocarps for germinating should not be collected until there have been one or two sharp frosts. The sporocarps should be allowed to dry gradually, after which they may be kept in a box until needed for use. They seem to retain their power of germination almost indefinitely. Sporocarps from poisoned herbarium material thirty years old have germinated readily. Even sporocarps which had been preserved in 95 per cent. alcohol for several years have been known to germinate.

FIG. 76.—*Marsilea quadrifolia.* × 560

A, microspore before germination. *B*, microspore with antheridia nearly mature. Fixed in chromo-acetic acid and stained in safranin, gentian-violet, orange.

To germinate sporocarps, cut away a portion of the hard wall along the front edge and place the sporocarp in a dish of water. The gelatinous ring with its sori will sometimes come out in a few minutes. In less than 24 hours the microspores, starting from the one-cell stage, will produce the mature antherozoids. The development of the megaspore is equally rapid. Embryos are abundant in 2 or 3 days. To secure a series of stages in the development of the gametophytes and embryo, it is necessary to fix material at short intervals. For morphological work, picro-acetic acid, used hot (85° C.), is very good, since the material does not occasion so much difficulty in cutting.

The fixing agent should be hot when the material is put into it, but it should not be kept hot. Allow it to cool immediately. Five or 6 hours will be sufficient.

Dr. F. M. Lyon found a modification of Carnoy's fluid (glacial acetic acid 2 parts, absolute alcohol 1 part, and chloroform 1 part) a good fixing agent for the spores of *Selaginella*. The material should remain in the fixing agent 1 or 2 hours or until it begins to become transparent. Wash in absolute alcohol, and pass through the mixtures of alcohol and xylol, following the schedule given in the next paragraph. Chromo-acetic (1 g. chromic acid, 2 c.c. glacial acetic acid, 200 c.c. water) material allows better staining, but the cutting is more uncertain. It is best to prick the megaspores with a needle while they are in the fixing fluid, in order to facilitate the infiltration of paraffin. Better mounts of the microspores can be obtained if the troublesome megaspores be picked out from the sorus while the material is still in the fixing agent or the alcohols. The megaspores must be imbedded in rather hard paraffin, and one must expect to hone the knife thoroughly before it can be used again, for when the knife strikes a megaspore of one of the heterosporous pteridophytes, it seems like striking a grain of sand.

The following schedule will usually give good sections of the hard megaspores of the heterosporous pteridophytes, whether they are to be cut separately or in their sori or strobili. The long periods are neither necessary nor desirable for the younger stages, which cut readily after the usual shorter periods; but when the megaspore walls become hard, the usual periods fail to secure proper infiltration. The method is essentially that used by Dr. F. M. Lyon in her work on *Selaginella*:

1. Chromo-acetic acid, 1 to 3 days.
2. Wash in water, 1 day.
3. 15, 35, 50, 70, 85, and 95 per cent. alcohol, 1 day each.
4. Absolute alcohol, 2 days, changing several times.
5. Mixtures of alcohol and xylol, 2 days.
6. Pure xylol, 1 day.
7. Add to the xylol as much paraffin as will dissolve at the room temperature; after 2 or 3 days, put the bottle on a board on the top of the bath where the temperature will be about 25° to 30° C. Allow the material to remain here 3 or 4 days, adding as much paraffin as will go into solution. Then put the bottle on the bath where the

temperature will be 35° to 45° C., and continue to add paraffin. The material should remain here for a week. If the temperature does not rise higher than 35° C., it will be an advantage to leave material here for a month.

8. Put the material in the bath in a watch-glass, or any dish with considerable surface, in order that the xylol may be driven off rapidly. Only 1 or 2 hours will be needed for complete infiltration. The paraffin should be changed 2 or 3 times.
9. Imbed in rather thin cakes.

While the method is tedious, it is worth the trouble, for even old strobili of *Selaginella* yield smooth ribbons at 5 μ, with paraffin no harder than 54°.

As Fig. 66 suggests, the mature archegonia, and especially the young embryos, may be removed from the top of the megaspore and cut with perfect ease.

The spermatozoid, which in *Marsilea* has an unusually large number of turns in the spiral, is easily mounted whole. When the spermatozoids have become numerous, put several megaspores upon a slide and heat gently until dry. Then wet the preparation in any alcohol and stain sharply in acid fuchsin. Dehydrate, clear in clove oil, and mount in balsam. Such a preparation will often show a score of spermatozoids in the gelatinous funnel leading down to the neck of the archegonium.

CHAPTER XIX

PTERIDOPHYTES

EQUISETALES

It is not difficult to secure prothallia of *Equisetum*. They may be grown by the method already described for the *Filicales*, or the spores may be sown in various pots in the greenhouse. The soil in the pots must be kept damp, but not too wet. The spores must be sown as soon as they are shed, or they will not germinate. Antheridia begin to appear in about a month, the archegonia appearing somewhat later. If the plants are too crowded, as is likely to be the case, only antheridial plants may be secured. In the vicinity of Chicago the spores of *Equisetum arvense* are shed during the latter part of April, but spores of other species may be obtained in the summer.

The prothallia are so small that for morphological purposes it is better to mount them whole. With a knife, skim off a thin layer of soil, just thick enough to hold the prothallia together. Fix in chromo-acetic acid and mount by the Venetian turpentine method. In staining with Magdala red and anilin blue, care must be taken not to stain too deeply in the blue. Iron-hæmatoxylin or Delafield's hæmatoxylin may be used after the washing in water.

For sections, the dirt and sand must be removed with great care, either before fixing or during the washing in water. Sections should not be thicker than 5μ. The safranin, gentian-violet, orange combination can be recommended for the devolopment of antheridia and spermatozoids.

The sporangia are harder to cut, but good preparations should be secured from paraffin material. *E. arvense* is abundant everywhere, and is to be preferred on account of the comparative ease with which the sporangia and other portions of the fertile shoot can be cut. Longitudinal sections of the younger strobili show various stages in the development of the spores, the more advanced

stages being found at the base of the strobilus. Tetrads may be found at the base of the strobilus, while the spore mother-cells at the apex are still undivided. Of course, it is impossible to stain a longitudinal section of such a strobilus so that all stages will be satisfactory. For the beginner, at least, this is not a serious objection, for he will be almost sure to secure some stage beautifully stained. The experienced worker, who is able to control his staining with more precision, will prefer transverse sections. Material for sporangia should be obtained as soon as the fertile shoot appears above ground, and if it can be obtained earlier, so much the better. When the spores are shed, the young sporangia which are to develop the next year can already be detected. The safranin, gentian-violet, orange combination can be recommended for the development of the mother-cell and the formation of tetrads, but Delafield's hæmatoxylin or Haidenhain's iron alum-hæmatoxylin will be more satisfactory for earlier stages.

The roots are very small, but have large cells and easily yield good preparations. If a handful of *Equisetum limosum* or *E. hiemale* growing in water be pulled up, scores of root-tips may be secured in a few minutes. In case of such small objects it is a good plan to add a few drops of eosin to the alcohol during the process of dehydrating, in order that the material may be seen more easily. The slight staining does no damage, even if more critical stains are to be used after the sections are cut. Longitudinal sections of the roots may also be obtained by cutting transverse sections of the nodes. Sections of the stem of the fertile shoot of *E. arvense* are easily cut in paraffin or celloidin, but sections of the stem of *E. hiemale* or similar species do not cut in paraffin. Jeffrey's modification of the celloidin method should be used for older stems, and the desilification should be particularly thorough. The growing points of stems, however, may be cut with ease in paraffin. *E. arvense* is particularly favorable on account of the numerous apical cells which may be found in a single preparation. Delafield's hæmatoxylin, used alone, is good for the apical cells, but for sections of older stems a slight counter-stain with erythrosin will improve the mount.

CHAPTER XX

PTERIDOPHYTES

LYCOPODIALES

Lycopodium.—The prothallia of some species of *Lycopodium* are subterranean and tuberous like those of *Botrychium* and *Ophioglossum*, while the prothallia of other species are partly subterranean and partly aërial, the aërial portions, which are green, bearing the antheridia and archegonia. Attempts to raise prothallia from spores are uniformly unsuccessful, perhaps because the presence of the endophytic fungus is necessary to any development. Very few have ever succeeded in finding prothallia in the field. When found, they are perfectly easy to cut and stain.

Before fixing a strobilus of *Lycopodium*, a slab should be cut from both sides. If the strobilus is simply halved lengthwise, both pieces curve so that it is impossible to get the best sections. The strobili of *L. inundatum* and *L. lucidulum* are easier to cut than those of drier forms like *L. clavatum* and *L. obscurum*. Transverse sections do not give good views of the sporangia. A piece of the strobilus of *L. inundatum* three-fourths of an inch in length will show a fine series of stages from the origin of the archesporium to the division of the spore mother-cells. Since there are no megaspores with their hard walls, the technique presents none of the difficulties of the heterosporous forms.

Good sections of the stem and rhizome can be made from fresh material without any imbedding. The best results are secured by the celloidin method. The structures, except quite near the apex, are too hard to cut in paraffin.

Psilotum.—The young sporangia should be cut in paraffin, but the older ones should be treated like the megaspores of heterosporous forms. The stem should be imbedded in celloidin, and even the mature sporangia are likely to yield better sections by this method.

Selaginella.—The prothallia of *Selaginella* develop within the spores while the spores are still within the sporangia. Consequently, sections of prothallia may be secured by cutting sections of the strobili. Strobili with sporangia in early stages of development cut easily by the usual methods, but when the walls of the megaspores become hard, Dr. F. M. Lyon's method, described at the close of the preceding chapter, should be used. Many species of *Selaginella*, including common greenhouse forms, have strobili which are square in transverse section. When imbedded, these naturally lie on a flat surface, and the beginner is likely to cut parallel with this surface, thus securing very few good views of the sporangia. Such strobili should be cut longitudinally so that the sections pass from one corner to the corner diagonally opposite. This will give good longitudinal sections of two rows of sporangia. (Fig. 68, *A*.)

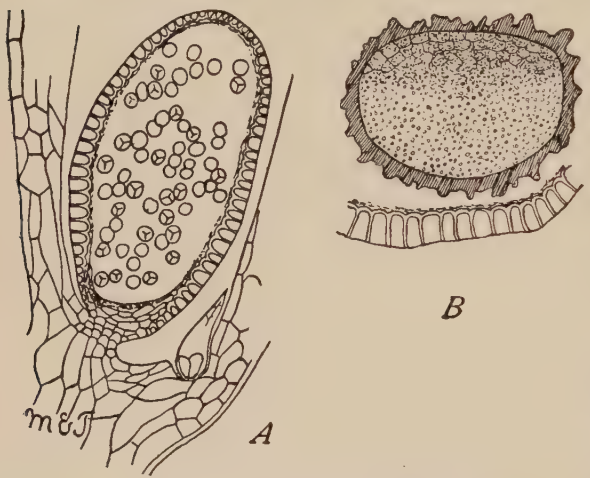

FIG. 68.—*Selaginella Mertensii*. × 93

A, microsporangium containing microspores. *B*, a megaspore showing the beginning of the prothallium. Fixed in picro-acetic acid and stained in Delafield's hæmatoxylin. (From a drawing by Miss M. E. Tarrant.)

The older megaspores had better be cut one at at time. Pick them out and prick them with a needle. A slight puncture at the basal portion of the megaspore does no damage and insures a thorough infiltration. If the megaspores are imbedded separately, they will usually orient themselves so that sections perpendicular to the paraffin cake will show the most instructive views of the gametophyte structures.

For archegonia, fertilization, and young embryos, prepare the

soil as directed for fern prothallia; lay upon the soil a frond in which the megaspores have reached their full size. The development is not rapid. The spores drop off, and development continues. The condition may be examined from time to time with a pocket lens. When the triradiate crack at the apex of the megaspore becomes visible, archegonia, or some stage in the development of the embryo will be found.

Interesting material for demonstration can be prepared by putting strobili or pieces of fronds with numerous strobili into a mixture of equal parts of 95 per cent. alcohol and glycerine. The various positions of megasporangia and microsporangia in the strobilus is easily seen with the naked eye, since the megasporangia appear yellow, while the microsporangia are reddish in color.

The distribution will be found to depend, in some measure, upon the position of the frond.

Isoetes.—*Isoetes* is easily kept all the year in the laboratory. Put about three inches of soil in the bottom of a tall two-gallon glass jar. Set out the plants and fill the jar two-thirds full of water. The jar should be kept covered with a piece of glass, if there is any danger from bacteria or other pests. Such a jar will support half a dozen large plants. In the dirt and in the decaying sporangia around the bases of old plants, ripe spores may be found. Developing prothallia are often found in these spores. Further stages are more easily watched by transferring such spores, with only a small quantity of dirt, to a shallow dish of water.

The microsporangia cut easily. The megasporangia are difficult, but not as difficult as those of *Selaginella*. For older stages, it is better to cut one sporophyll at a time. The sporophyll should be cut off just above the tip of the ligule and just below the lower portion of the velum. The inner portion with numerous younger sporangia, may be cut entire. Iron-hæmatoxylin is good for younger stages, but the safranin, gentian-violet, orange combination is better for the mother-cell and its divisions. Cyanin and erythrosin is a particularly good combination for the mature megaspores.

The stem of *Isoetes* is particularly interesting on account of certain resemblances to the stems of Dicotyls. Even the larger stems an inch in diameter cut well in paraffin. Smaller stems show essentially the same structures as the larger ones, and more sections can be mounted upon a single slide. To make the most instructive preparations, the whole stem should be cut transversely, and sections from various portions of the ribbon should be mounted upon each slide. Safranin and Delafield's hæmatoxylin is a satisfactory combination.

CHAPTER XXI

SPERMATOPHYTES

In this immense group we cannot hope to give even approximately complete directions for making preparations, but must be content to give a few hints which may prove helpful in collecting material and in securing mounts of the more important structures of the flowering plants. We shall consider the gymnosperms and the angiosperms separately, although in many respects the technique is the same for both.

GYMNOSPERMS

Since *Pinus* is an available laboratory type, we shall describe methods for demonstrating various phases in the life-history of this genus, hoping that the directions will enable the student to experiment intelligently with other forms. Except when other forms are definitely mentioned, the following directions apply particularly to *Pinus*.

Spermatogenesis.—In October the clusters of staminate cones which are to shed their pollen in the coming spring are already quite conspicuous. The cones should be picked off separately, and the scales should be carefully removed so as to expose the delicate greenish cone within. At this time the sporogenous cells are easily distinguished. Material collected in January, or at any time before growth is resumed in the spring, shows about the same stage of development. If it is desired to secure a series of stages with the least possible delay, a branch bearing numerous clusters of cones may be brought into the laboratory and placed in a jar of water. Growth is more satisfactory in case of branches broken off in the winter than in those brought in before there has been any period of rest. The material can be examined from time to time, and a complete series is easily secured. The mitotic figures in the pollen mother-cells furnish exceptionally instructive preparations. The two mitoses take place during the last week in April

and the first week in May. Staminate cones which will yield mitotic figures can be selected with considerable certainty by examining the fresh material. Crush a microsporangium from the top of the cone and one from the bottom, add a small drop of water and a cover to each, and examine. If there are pollen tetrads at the bottom, but only undivided spore mother-cells at the top, it is very probable that longitudinal sections of the cone will yield the figures. If a drop of methyl green be allowed to run under the cover, it will enable one to see whether figures are present or not. When desirable cones are found, they should be halved longitudinally or slabs should be cut from the two sides, in order that the fixing agent may penetrate more rapidly and that infiltration with paraffin may be more thorough.

The later stages, showing the germination of the microspores, furnish better sections if the cones are cut transversely into small pieces about three-sixteenths of an inch thick. It is very easy to get excellent mounts of the pollen just at the time of shedding, which, in *Pinus Laricio* in the vicinity of Chicago, occurs near the middle of June. Shake a large number of cones over a piece of paper, thus securing an abundance of material. Fix in chromo-acetic acid, wash in water (a few minutes is sufficient, and the water need not be changed), pass through the alcohols, allowing each to act for about 2 hours, make the usual gradual transition from alcohol to xylol, and from xylol to paraffin. Infiltration in the bath will not require more than 1 or 2 hours. When the infiltration is complete, there should be only enough paraffin to cover the mass of pollen grains. The material may now be poured out into a rectangular dish or box with surface enough to make the cake about one-eighth of an inch thick. Good results may be secured by pouring the paraffin upon a cold piece of glass. Another method is to keep the material in a small bottle during infiltration, and when ready to imbed, simply cool the bottle. Break the bottle carefully, cut off the lower portion of the paraffin containing the pollen, mount it on a block in the usual manner, and trim away some of the paraffin so that two parallel surfaces will make the sections ribbon well. Sections should not be thicker

than 5μ. Material in this stage shows a large tube nucleus, a somewhat lenticular cell with a more deeply staining nucleus, and, lastly, two small prothallial cells quite close to the spore wall. The prothallial cells cannot always be detected at this stage, and there may be some doubt as to whether two such cells are always present. The division of the lenticular cell into "stalk cell" and "body cell," and also the division of the body cell into the two male cells, must be looked for in sections of the nucellus of the ovule.

Oögenesis.—In *Pinus Laricio* the rudiment of the ovulate strobilus, which is to be pollinated in June, can be detected in the preceding October. The collection of this stage is very uncertain, because there seems to be no mark distinguishing buds containing ovules from buds which are only vegetative. By collecting numerous buds from the tops of vigorous trees which are known to produce an abundance of strobili, a few buds containing the desired stages may be obtained. In May, after the strobili break through the bud scales, material is easily collected. Up to the time of pollination the entire ovulate strobilus cuts easily in paraffin. Longitudinal sections of the cone at this time give good views of the bract and ovuliferous scale bearing the ovules. The integument is very well marked, and in the nucellus one or more sporogenous cells can usually be distinguished. As soon as the scales close up after pollination, the cone will be too hard to cut, and it will be necessary to remove the scales and cut them separately. For a study of the ovule and the structures within it, better preparations will be obtained by carefully cutting off the pair of ovules from the scale. For preparations like that represented in Fig. 69, *A*, it is a good plan to remove the endosperm with its archegonia from the ovule. Fixing, infiltration, and cutting will then occasion but little trouble, and the whole ribbon may be got upon a single slide. However, at this stage the pollen tubes with their contents are rapidly working their way through the nucellus toward the archegonia, and consequently it is better to retain enough of the tissues of the ovule to keep the nucellus in place. In later stages, after fertilization has taken

place, it is necessary to remove the endosperm. In stages like Fig. 69, *B*, *C*, *D*, and later, the developing testa should be dissected away with great care, for a very slight pressure is sufficient to injure the delicate parts within. Mature embryos may be dissected out from the endosperm before fixing, but it is hardly

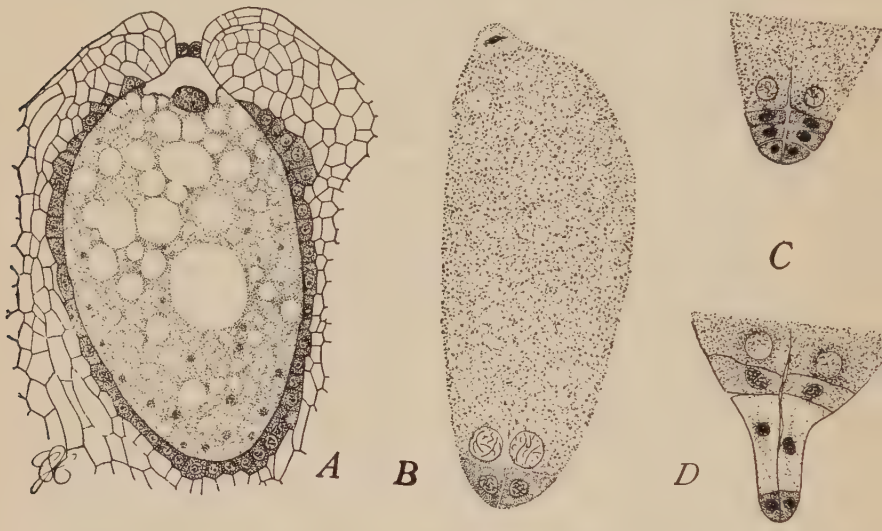

FIG. 69.—*Pinus Laricio*. × 104

A, top of prothallium with an archegonium just before the cutting off of the ventral canal cell. Fixed in Flemming's weaker solution and stained with Haidenhain's iron alum-hæmatoxylin. Collected June 18, 1897. *B*, *C*, and *D*, early stages in the formation of the embryo. Fixed in chromo-acetic acid, and stained in safranin, gentian-violet, orange. Collected July 2, 1897.

necessary, since they cut quite well if left in place. The "pine nuts" or "piñon," to be found upon the market, are good for a study of the mature embryo. The testa, which is quite a hard shell, should be taken off, and the endosperm should be allowed to soak in water for about 24 hours, after which the embryo may be dissected out and fixed.

The period at which the various stages may be found varies with the species, the locality, and the season. In *Pinus Laricio* (the common Austrian pine) the megaspore mother-cells appear as soon as the young strobili break through the bud scales. At Chicago, in the season of 1897, material collected May 27 did not

yet show archegonia; the ventral canal cell was cut off about June 21 (see Fig. 70), the fusion of the pronuclei occurred about a week later, and stages like Fig. 69, *B*, *C*, and *D*, were common in material collected July 2. In the season 1896 all the stages appeared about two weeks earlier. In *Pinus sylvestris* the stages appeared a little earlier than in *Pinus Laricio*.

After the stage shown in Fig. 69, *A*, has appeared, it is necessary to collect at intervals of not more than two days until the stage shown in Fig. 69, *D*, is reached. If collections are made at intervals of 4 or 5 days, the most interesting stages, like the cutting off of the ventral canal cell, fertilization, and the first divisions of the nucleus of the oöspore, may be missed altogether. It should be mentioned that all the ovules of a cone will be in very nearly the same stage of development.

For all stages in the life-history of *Pinus*, chromo-acetic acid is a successful fixing agent. The following is a good formula:

Chromic acid	2 g.
Glacial acetic acid	6 c.c.
Water	200 c.c.

If there should be any plasmolysis during the stage of free nuclear division in the endosperm, decrease the proportion of chromic acid.

The addition of 10 drops of 1 per cent. osmic acid to 50 c.c. of the above solution accelerates the killing and thus increases the number of mitotic figures, but does not cause any disagreeable blackening. The popular Flemming's solutions often fail to give as good results as the cheaper fixing agent.

The following stains may be suggested: for studying the pollen tubes in the nucellus, cyanin and erythrosin; for the development of the archegonium up to the stage shown in Fig. 69, *A*, Delafield's hæmatoxylin; for the stages shown in Fig. 69, *A*, iron alum-hæmatoxylin or the safranin, gentian-violet, orange combination; for the stage shown in Fig. 70, nothing seems to equal the safranin, gentian-violet, orange combination; for stages like Fig. 69, *B*, *C*, and *D*, and also for later stages in

the development of the embryo, Delafield's hæmatoxylin brings out the walls perfectly, but since mitotic figures are very frequent in these stages, it is worth while to use the safranin combination with some preparations, although it is much inferior to Delafield's hæmatoxylin when cellulose walls are to be emphasized. The fully developed embryo surrounded by the endosperm, as it is found from September until it is shed the following spring, yield elegant preparations when stained in cyanin and erythrosin.

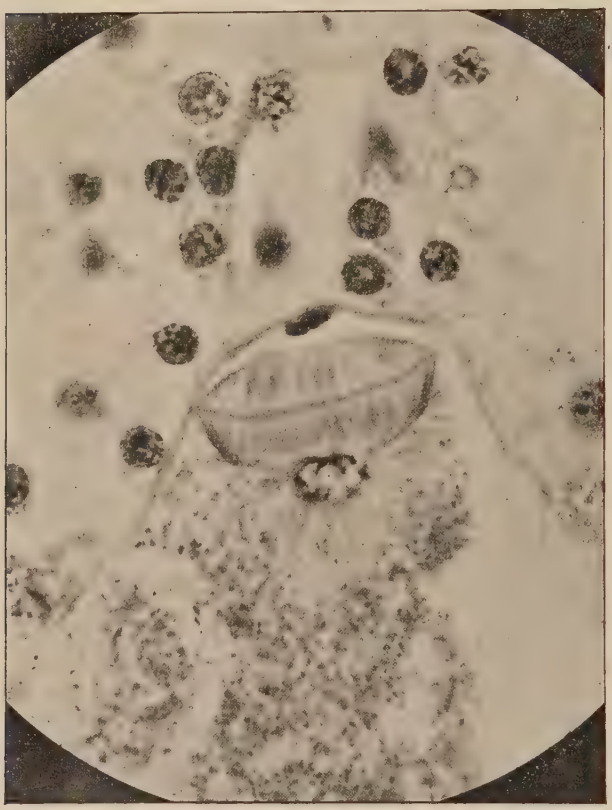

FIG. 70.— *Pinus Laricio*. × 710

The mitotic figure concerned in cutting off the ventral canal cell. The nucleus at the lower end of the spindle is the nucleus of the oösphore. Fixed in chromo-acetic acid, and stained in the safranin, gentian-violet, orange combination. Collected June 21, 1897. Negative by Dr. W. H. Knap from a preparation by the author.

All the stages which have been described can be cut in paraffin with little difficulty.

The Leaves.—The leaves of our common gymnosperms cut readily in paraffin while they are young and tender, but as they approach maturity it is a fruitless task to attempt paraffin sections.

Celloidin sections are far more satisfactory. Cut the needles into pieces about one-fourth of an inch long, fix in a picro-corrosive-acetic mixture ($\frac{1}{2}$ g. picric acid, 2 g. corrosive sublimate, 1 c.c. glacial acetic acid, 100 c.c. 50 per cent. alcohol). If used hot, 5 minutes is sufficient, but if used cold it should be allowed to act for 2 or 3 hours. After the material has been imbedded in celloidin, the block should be placed in equal parts of 95 per cent. alcohol and glycerine for a few days, after which it should cut quite readily. Stain with safranin and Delafield's hæmatoxylin, clear in Eycleshymer's clearing mixture, and mount in balsam.

Fairly good sections may be obtained in great quantities with little trouble by the following method: Make a bunch of the needles as large as one's little finger, wrap them firmly together with a string, allowing about an eighth of an inch of the bunch to project above the wrapping; then fasten the whole in a sliding microtome or a hand microtome, and every stroke of the razor will give twenty or thirty sections, some of which will surely be good. As the sections are cut, they may be put directly into 95 per cent. alcohol, and after a few minutes can be transferred to 50 per cent. alcohol and then to the stain. Dehydrate, clear in xylol, and mount in balsam.

Stems and Roots.—With a sharp razor fairly good sections of stems and roots may be made without imbedding, especially if the microtome be used. Young buds may be cut in paraffin. Stems and roots as large as half an inch in diameter can be cut in celloidin. The material should be cut into pieces not more than one-fourth of an inch long. The following treatment should give good results:

1. Picro-corrosive-acetic mixture, 5 minutes if used hot, or 2 to 3 hours if used cold.
2. Wash 1 day in 50 per cent. alcohol, to which a little iodine has been added; 70 per cent. alcohol, one day. (At this point it will be an advantage to use hydrofluoric acid as recommended by Jeffrey. Directions are given in the chapter on the celloidin method.) 85, 95, and 100 per cent. alcohol, 1 day

each ; change the 100 per cent. alcohol and allow it to act for 5 or 6 hours longer.

3. Ether-alcohol, 24 hours. Some prefer to precede the ether-alcohol by a mixture of equal parts of ether-alcohol and absolute alcohol.
4. Thin celloidin (about 2 per cent.), 2 to 3 days ; 6 per cent. celloidin, 2 to 3 days ; 10 per cent. celloidin, 2 to 3 days. After the thin celloidin has acted for a few days, the cork may be removed for a short time each day, thus allowing the thin celloidin to become thick by the evaporation of the ether-alcohol.
5. Get some small blocks of wood (three-eighths inch cubes of white pine are good), wet one of them in ether-alcohol, dip it into thin celloidin, place the object upon the block in convenient position for cutting, pour over it a few drops of 10 per cent. celloidin, and then plunge the whole into chloroform. Leave it in the chloroform about 24 hours, and then transfer to a mixture of equal parts of 95 per cent. alcohol and glycerine, where it should remain for several days. Material may be kept here indefinitely. Even refractory stems may be cut after they have been in this mixture for a couple of weeks.
6. Cut the sections, keeping the knife wet with alcohol and glycerine mixture. Wash the glycerine out in 50 per cent. alcohol, and transfer the sections to the stain.
7. Stain in safranin, 24 hours ; wash for about 1 minute in 50 per cent. alcohol, and then stain in Delafield's hæmatoxylin 5–10 minutes ; acid alcohol, 2–10 seconds ; wash the acid out in 70 per cent. alcohol ; 95 per cent. alcohol, 5 minutes ; absolute alcohol until the celloidin matrix is largely removed ; clove oil until all the celloidin is removed ; mount in balsam.

Xylem should show a brilliant red color and cellulose a rich purple, if the stain is successful. If either stain is too weak or too prominent, the duration of the stain, the length of time in the alcohols, or the time in acid alcohol must be varied until the desired result is secured.

People who make all their anatomical sections without imbedding may regard this method as tedious and unnecessary, but such preparations will show much which is never seen in mere free-hand sections, for the reason that free-hand sections, if thin enough to show any detail, will lose most of their cell contents, while in celloidin sections everything is held in place. Even if the celloidin sections be passed through absolute alcohol and cleared with clove oil, a process which dissolves away the celloidin, the contents of the cells will still be retained in most cases, and stains which cannot be extracted from celloidin may be used.

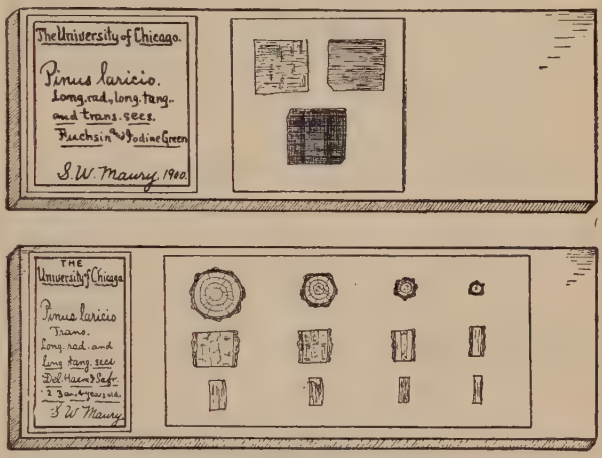

FIG. 71

Slides showing labels and methods of arranging sections.

For preparations of the mature wood a piece of white pine from a dry-goods box furnishes perfect material. It should not be imbedded, but the cutting will be facilitated if the piece be soaked for a few hours in water or in the alcohol and glycerine mixture. In all preparations designed to show the structure of wood there should be three sections—a transverse, a longitudinal radial, and a longitudinal tangential. These may be arranged as in the upper slide of Fig. 71, but if it is desired to make a thorough study of the structure, it is a good plan to have on each slide several sections of each kind, thus having an opportunity to use a variety of stains. Fuchsin and methyl green is a good combination. The safranin and Delafield's hæmatoxylin is also excellent.

For a study of young stems or roots, preparations like that shown in the lower slide of Fig. 71 will be found very convenient.

The Cycads and Ginkgo.—Material of *Ginkgo* is becoming somewhat accessible. Pollen is shed about May 10, and fertilization takes place late in September. The entire staminate strobilus is not at all difficult to cut in paraffin, even when mature. For the pollen-tube structures a small portion of the nucellus should be removed and treated separately. Safranin and gentian-violet seems to be the best combination for blepharoplasts and other structures within the pollen tube.

The staminate strobili of the Cycads are so large that they can be cut entire only in the youngest stages. For nearly all stages the sporophylls must be removed. The outer ends of the sporophylls, forming the surface of the strobilus, soon become too hard to cut. It is better to remove this harder portion and fix only the part bearing the sporangia. For small sporophylls, like those of *Zamia*, this is sufficient. Where the sporophylls are large, as in *Cycas* and *Encephalartos*, it is better to cut off from the surface of the sporophyll a thin slice bearing the sporangia.

As soon as the integument is removed, the position of the pollen tubes is readily determined, since the haustorial portions of the tubes form conspicuous brownish lines radiating from the nucellar beak. Cut off a portion of the nucellus, including all the brownish lines. In cutting, the knife should strike the outer portion containing the brownish lines. Safranin and gentian-violet, with or without orange, is an excellent stain for all stages in the development of the male gametophyte.

The ovules of the Cycads and *Ginkgo* are very large, and, when mature, thin sections cannot be cut by any method yet discovered. In younger stages it is not difficult to get good sections of the entire ovule. Slabs should be cut from two sides of the ovule to facilitate fixing and infiltration. During free nuclear stages in the endosperm, and even during earlier stages in the formation of walls, care must be taken that the slabs may not cut into the endosperm, which is so turgid that distortion would be sure to result. Even after the ovule approaches its full size, it can be cut entire, until the stony layer begins to harden. When the fresh ovule can no longer be halved easily with a razor, it is

not worth while to try to cut it in paraffin. Instructive preparations may be made by cutting from the median longitudinal portion of the ovule a slab about 5 mm. thick. The slab should be fixed, washed, dehydrated, and cleared in xylol. It should then be kept in a flat-sided bottle. Such a preparation shows the integument, micropyle, nucellus with its beak, pollen tubes, the stony and fleshy layers, general course of vascular bundles, and the female gametophyte with its archegonia.

For thin sections of the archegonia, a cubical piece with an edge of 6 or 8 mm. should be cut from the top of the endosperm with a very sharp, thin blade. The slightest pressure upon the archegonia will ruin the preparations.

After the embryos begin to grow down into the endosperm, oblong pieces containing the embryos should be cut out.

After the cotyledons appear, useful preparations may be made by dissecting out the entire embryos, which may be fixed in chromo-acetic acid, washed, stained in eosin or in Delafield's hæmatoxylin, placed in 10 per cent. glycerine, and mounted in glycerine jelly or by the Venetian turpentine method. Since the suspensors become long and irregular, each embryo should be placed in a separate dish, lest the suspensors become entangled and broken.

After the stony layer becomes hard, it is better to use a small fret saw for opening the ovule. Before the embryo has pushed down into the endosperm, the ovule should be sawed in two transversely. The endosperm and nucellus can then be picked out and treated as desired. After the tip of the embryo reaches the middle of the endosperm, the ovule should be sawed open longitudinally.

CHAPTER XXII

SPERMATOPHYTES

ANGIOSPERMS

Success depends largely upon judgment and care in selecting and trimming material before it is put into the fixing agent in the field. While the following directions cannot be applied to all plants, they should, nevertheless, enable the student to make such modifications as may be demanded by any particular form.

Floral Development.—For a study of floral development very young buds are necessary, and it is best to select those forms which have rather dense clusters of flowers, in order that a complete series may be obtained with as little trouble as possible.

The usual order of appearance of floral parts is (1) calyx, (2) corolla, (3) stamens, and (4) carpels; but if any of these organs is reduced or metamorphosed, their order of appearance may be affected.

Floral development is easily studied in the common *Capsella bursa-pastoris*. The best time to collect material is late in March or early in April. Dig up the plant, carefully remove the leaves, and in the center of the rosette a tiny white axis will be found. A series of these axes from one-eighth of an inch to three-eighths of an inch in length, and from one-sixteenth to three-sixteenths of an inch in diameter will give a very complete series of stages in the development of the floral organs. Preparations from the apex of the shoot taken after the inflorescence appears above ground are not to be compared with these taken early in the season, because the pedicels begin to diverge so early that median longitudinal sections of the flowers are comparatively rare. Fix in chromo-acetic acid and stain in Delafield's hæmatoxylin. The sections should be longitudinal and about 5 μ thick.

In the sweet clover, *Melilotus alba*, it is much easier to secure material because the spikes of developing flowers appear after the

plant is above ground. *Prunus* and many other members of the Rosaceae furnish examples of the perigynous type of development.

The common dandelion, *Taraxacum officinale*, affords an excellent series with little labor. Examine vigorous plants, which have, as yet, no flowers or buds in sight. Dig up the plant and dissect away the leaves. If there is a white cluster of flower buds, the largest not more than three-sixteenths of an inch in diameter, cut out the cluster, leaving only enough tissue at the base to hold the buds in place. Larger heads should be cut separately. Fix and stain as in *Capsella*.

Our most common thistle, *Cnicus lanceolatus*, shows the floral development with unusual clearness, but the preparation of the material is somewhat tedious. The involucre, which is too hard to cut, must be carefully dissected away. Retain only enough of the receptacle to hold the developing florets in place. A series of sizes with discs varying from one-eighth of an inch to three-eighths of an inch in diameter will show the development from the undifferentiated papilla up to the appearance of the archesporial cell in the nucellus of the ovule. The Canada thistle, *Cnicus arvensis*, is equally good, but it is more difficult to dissect out the desirable parts. In the common sunflower, *Helianthus annuus*, the young floral parts, like the mature head, are so very large that a satisfactory study may be made with a low-power objective. As in case of the thistle, the involucre must be trimmed away and only enough of the receptacle retained to hold the florets together.

In the willows, *Salix*, the bud scales must be removed and the copious hairs should be trimmed off as much as possible with scissors, after which the catkin should be cut in two longitudinally and placed in the fixing agent.

The cat-tail, *Typha*, presents a simple type of floral development. The leaves should be dissected away long before the flowers can be seen from the outside. The cylindrical clusters, varying in diameter from 2 or 3 mm. up to the size of one's finger will afford a complete series of stages. Until the spadix reaches the diameter of a lead pencil, transverse sections are easily cut. For later stages, the outer part of the spadix should

be sliced off so that only enough spadix is retained to hold the florets in place.

Spermatogenesis.—The earlier stages in spermatogenesis will be found in the preparations of floral development. The origin of the archesporium, the origin of sporogenous tissue, and the formation of the tapetum are beautifully shown in longitudinal and in transverse sections of the anthers of *Taraxacum* and many other Compositae. Transverse sections of the head of *Taraxacum* or any similar head at the time when pollen mother-cells are rounding off in the center of the head, will show various stages from the mother-cells in the center to the tetrads of spores at the periphery. Transverse sections of the anther of *Polygala* give exceptionally well-defined views of the sporogenous areas.

Lilium, *Galtonia*, *Iris*, *Tradescantia*, and *Podophyllum* can be recommended for demonstrating the nuclear changes involved in the formation of spores from the mother-cell. Several species of *Lilium* are common in greenhouses, and these may be used where wild material is not available. In early stages where the sporogenous cells have not yet begun to round off into spore mother-cells it is sufficient to remove the perianth, retaining just enough of the receptacle to hold the stamens in place. Transverse sections show the six stamens and also the young ovary. After the spore mother-cells have begun to round off, each stamen should be removed so as to be cut separately. In securing the desirable stages showing the division of the mother-cell in microspores, much time and patience will be saved by determining the stage of development before fixing the material. Mitosis is more or less simultaneous throughout an anther. Long anthers are particularly favorable, since they may show a very closely graded series of the various phases of mitosis. An anther of *Iris* may show mother-cells with nuclei in synapsis at the top, while the mother-cells at the bottom have reached the equatorial plate stage of the first division; or, the mother-cells at the top may show the first division, while those at the bottom show the second. Determine the stage by examining a few mother-cells, before fixing.

From what has been said, it is obvious that longitudinal sections should be cut to show mitosis. Transverse sections should be cut to show the general structure of the anther. It is not necessary to cut the stamens into pieces before fixing, since they are easily penetrated and infiltrated; in later stages the stamens *must* not be cut into pieces, since the pollen grains are easily washed out.

The problem of fixing spore mother-cells has received much attention. In fixing mother-cells and the two mitoses by which spores are formed, investigators have used almost exclusively the chromo-osmo-acetic acid solutions of Flemming, some preferring the weaker solution and some the stronger. These solutions have been used in nearly all of the work of the Bonn (Germany) school. Osterhout[1] experimented with forty fixing agents, and then concluded that Flemming's stronger solution was the best. Professor Grégoire and his students have made this their principal fixing agent.

In spite of the weight of authority, we believe that the value of solutions with such a large proportion of osmic acid has been overestimated. The osmic acid undoubtedly accelerates the killing of the protoplasm. This is seen more readily in animals. If *Cyclops* be brought into 30 c.c. of the chromo-acetic acid solution recommended for fixing Gymnosperms (2 g. chromic acid, 6 c.c. glacial acetic acid, and 200 c.c. water), the animals will swim for a while; if 5 or 6 drops of 1 per cent. osmic acid to be added to the solution, the animals cease their movements almost instantly. Doubtless the osmic acid has the same effect upon plant protoplasm. Where fixing is slow, very few mitotic figures are found with the chromosomes midway between the equator and the poles. The addition of 10 drops of 1 per cent. osmic acid to 50 c.c. of the solution just mentioned will secure as large a proportion of anaphases as solutions which are stronger in osmic acid, and there is no disagreeable blackening.

[1] Osterhout, W. T. V., Cell Studies, I, Spindle Formation in Agave, *Proc. Cal. Acad. Sci. Botany.* Third Series **2**: 255–284. 1902.

Farmer and Shove[1] in studying these mitoses and also vegetative mitoses in *Tradescantia*, secured better results with a mixture of 2 parts of absolute alcohol and 1 part glacial acetic acid. They allowed the fixing agent to act 15–20 minutes, then washed in absolute and imbedded by the usual methods.

For early stages up to that shown in Fig. 63, *A*, Delafield's hæmatoxylin or iron-hæmatoxylin is very satisfactory; for stages between *A* and *D* of the same figure, the safranin, gentian-violet, orange combination and iron-hæmatoxylin are the most popular stains. These stains doubtless owe much of their effectiveness to the fact that they are so universally used, and that the methods

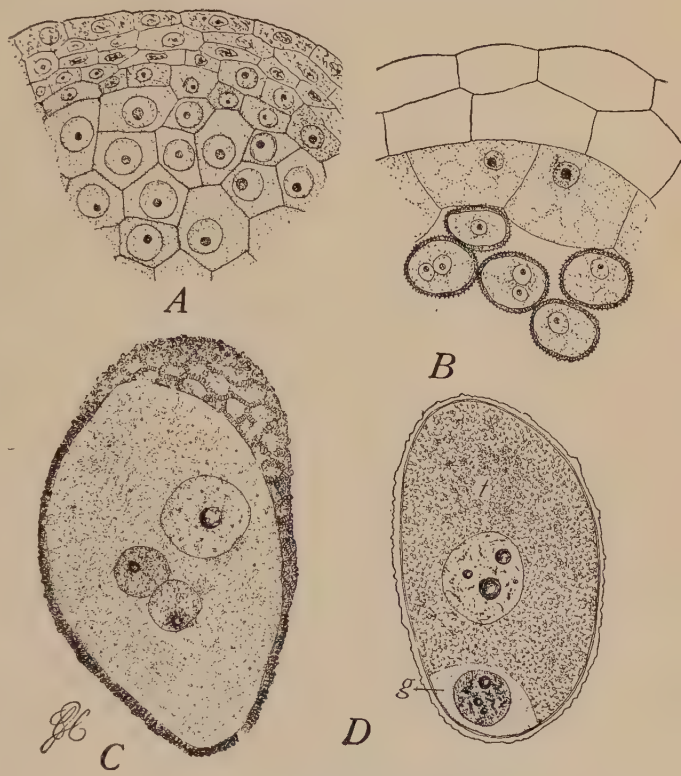

FIG. 72

A, *Salix tristis*. × 594. Transverse section of a portion of a young anther showing large sporogenous cells, one layer of tapetal cells (more deeply shaded in the figure), and from three to four layers of wall cells. *B*, *Salix petiolaris*. × 594. Small portion of transverse section of a nearly mature anther showing five pollen grains, two tapetal cells, and two layers of wall cells. *C*, *Lilium auratum*. × 505. Section of a pollen grain showing the large tube nucleus and two smaller generative nuclei. Fixed in chromo-acetic acid and stained in safranin, gentian-violet, orange. *D*, *Lilium tigrinum*. × 505. Pollen grain showing tube nucleus in the middle and a lenticular cell with the generative nucleus at one end of the grain.

[1] Farmer, J. B., and Shove, Dorothy, On the structure and development of the somatic and heterotype chromosomes of *Tradescantia virginica*, *Quart. Jour. Mic. Sci*, **48**: 559–569. 1905.

of applying them have become so highly perfected. In staining the mitoses in pollen mother-cells with the safranin, gentian-violet, orange combination, stain in safranin 6–24 hours; extract the stain with 50 per cent. alcohol until no color is left in the spindle. If this requires more than 10 minutes, a drop of hydrochloric acid may be added to the alcohol, but the acid must be washed out before proceeding. Dip the slide into water three or four times and then stain in gentian-violet 2–30 minutes. The time varies with different plants and with the condition of the material. If the gentian-violet acts too long, it will stain the chromosomes, and if too short, it will not stain the spindle properly. Dip in water, and then stain in orange 30 seconds. Dip in 95 per cent. alcohol, and then dehydrate rapidly in absolute alcohol. Clear in clove oil. Drain off as much of the clove oil as possible and then dip the slide in xylol or add a few drops of cedar oil, since the gentian-violet will fade if any clove oil remains. Examine in clove oil and make corrections if necessary. Mount in balsam. Do not add the balsam until the stain is satisfactory. Other combinations which are known to be successful are cyanin and erythrosin, acid fuchsin and iodine green, and Magdala red and anilin blue. In pollen grains at the stage in Fig. 72, *B*, *C*, and *D*, the tube nucleus stains red and the generative nucleus blue when cyanin and erythrosin are used. With Magdala red and anilin blue, the tube nucleus stains blue and the generative red.

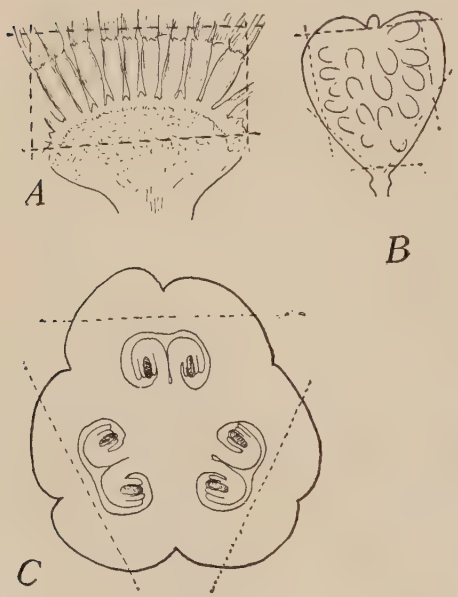

FIG. 73

A, head of *Aster*. *B*, pod of *Capsella*. *C*, transverse section of ovary of *Lilium*. The dotted lines show how the material should be trimmed before fixing.

The safranin, gentian-violet, orange combination shows nuclear details to better advantage, and readily distinguishes the tube and generative nuclei, although it does not give such a striking color contrast as the cyanin and erythrosin. The starch grains are brilliantly stained by the gentian-violet.

The mature pollen grain, after shedding, may be prepared by the method already described for *Pinus*.

Oögenesis.—As in spermatogenesis, the early stages will be found in preparations of floral development. The origin and development of the megaspore are easily traced in *Lilium*. In very young stages, before the appearance of the integument, the ovary may be removed from the flower and placed directly in the fixing agent, but in later stages, such as are shown in Figs. 66–71, strips should be cut off from the sides of the ovary in order to secure more rapid fixing and more perfect infiltration with paraffin. The dotted lines in Fig. 73, *C*, show about how much should be cut off. This is a much better plan than to secure rapid fixing and infiltration by cutting the ovary into short pieces, because the ovules will be in about the same stage of development throughout the ovary, and when one finds desirable stages like those from which these photomicrographs were taken, it is gratifying to have these pieces as long as possible.

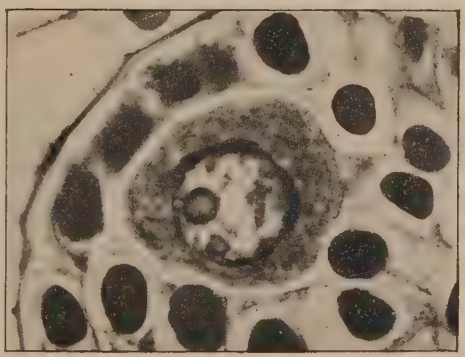

Fig. 74.—*Lilium philadelphicum*. × 710

Apex of nucellus contains a large archesporial cell with a large nucleus. In Lilium this archesporial cell becomes the embryo-sac directly without cutting off any tapetal cell or dividing into potential megaspores. Fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin.

Chromo-acetic acid, as just recommended for spermatogenesis (2 g. chromic acid, 6 c.c. glacial acetic acid, and 200 c.c. water) is equally good for oögenesis. Embryo-sacs at the fertilization period are likely to contain large vacuoles. In these stages with large vacuoles, the proportion of

acetic acid may be increased even up to 8 or 10 c.c. in order to avoid plasmolysis. The addition of 10 drops of 1 per cent. osmic acid is often an advantage. A much weaker solution (1 g. chromic acid, 6 c.c. glacial acetic acid, and 300 c.c. water), with the addition of the 10 drops of 1 per cent. osmic acid, has given excellent results.

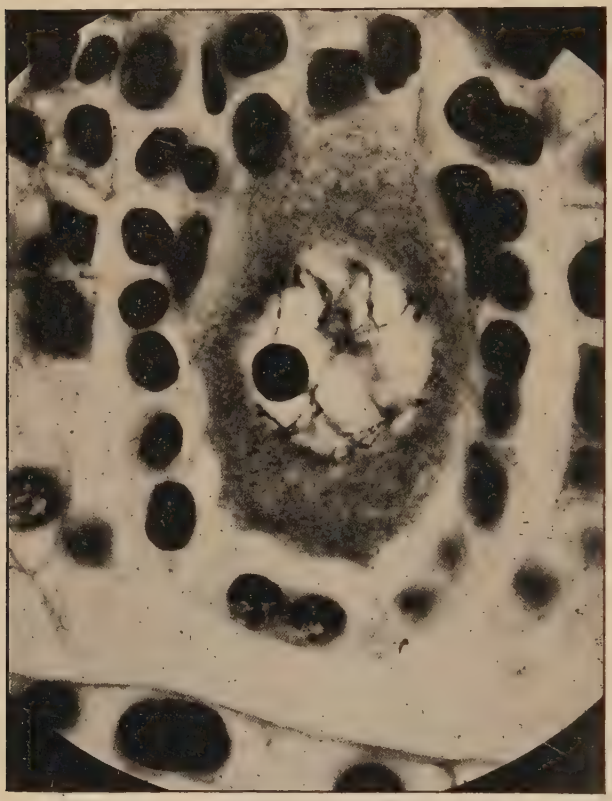

FIG. 75.—*Lilium philadelphicum*. × 710

Nucellus with megaspore. The chromatin thread in the nucleus is very distinct. Fixed in chromo-acetic acid and stained in safranin, gentian-violet, orange.

Lilium is an excellent form for demonstrating the stages shown in Figs. 74–80. The stage shown in Fig. 74 stains well in iron-hæmatoxylin or in Delafield's hæmatoxylin, but the later stages, shown in Figs. 75–80, stain better in the safranin, gentian-violet, orange combination. Stages like that shown in Fig. 75 and also those between Fig. 75 and Fig. 76 are the most difficult to stain, and only the utmost care and patience will insure first-class preparations. The thread of the nucleus in Fig. 75 shows a row of chromatin granules and soon becomes segmented into twelve chromosomes. If the

stain is too dense, the thread will probably appear smooth throughout all these stages, but if the staining is successful, the granules are sharply stained by the gentian-violet, while the linin thread is stained lightly or not at all. In successful staining with

FIG. 76 *Lilium philadelphicum.* × 710 FIG. 77

First division of the nucleus of the megaspore. (The same nucleus the earlier stages of which are shown in Figs. 65 and 66.) Fixed in chromo-acetic acid, and stained in safranin, gentian-violet, orange. Fifteen microns.

cyanin and erythrosin the granules stain blue and the linin red. Stages like Figs. 76 and 77 are easily stained, and the preparations are exceptionally beautiful. Stain for 24 hours in alcoholic safranin, and then rinse in 50 per cent. alcohol until the red color disappears from the spindle, but remains bright in the chromosomes and nucleoli; stain in gentian-violet 5–30 minutes; rinse in water about 30 seconds; stain in aqueous orange 15–30 seconds; dip in 95 per cent. alcohol; then transfer to

absolute alcohol, and move the slide back and forth in order to dehydrate as rapidly as possible (3–6 seconds will usually be long enough); the slide must be taken from the absolute alcohol while the gentian-violet is still coming out in streams; treat with clove

FIG. 78.—*Lilium philadelphicum.* × 710

Megaspore (embryo-sac) containing two daughter-nuclei resulting from the first division of the nucleus of the megaspore. A portion of the spindle still remains between the two nuclei. Fixed in chromo-acetic acid, and stained in safranin, gentian-violet, orange. Fifteen microns thick.

FIG. 79.—*Lilium philadelphicum.* × 710

Later stage in the development of the embryo-sac. Each of the two nuclei shown in Fig. 69 has divided. The nuclei are much smaller than those in Fig. 69. Fixed in chromo-acetic acid, and stained in safranin, gentian-violet, orange. Fifteen microns thick.

oil 10–30 seconds, and then drain off the clove oil and add a few drops of cedar oil, since the gentian-violet fades if much clove oil is left in the preparation. If the cedar oil is transparent and has a strong odor do not use it, but use xylol instead. Examine and, if the stain is satisfactory, mount in balsam. If the gentian-

violet has stained the chromosomes, treat again with clove oil; if the clove oil fails to remove the gentian-violet from the chromosomes, transfer to absolute alcohol. If this latter step should be necessary, it indicates that the staining in gentian-violet was too prolonged and should be shortened with the next preparation from the same material. Never put on the balsam and cover until the stain is satisfactory.

When the staining is properly done, the chromosomes will show a bright red color and the spindle a brilliant violet. If the gentian-violet is allowed to act too long or is poorly extracted, the chromosomes will appear violet or reddish-violet, and such details as the relation of spindle fibers to chromosomes will be obscured. Stages shown in Figs. 78 and 79 may be found in the same ribbons with stages like Figs. 76 and 77, and are all well differentiated by the same treatment.

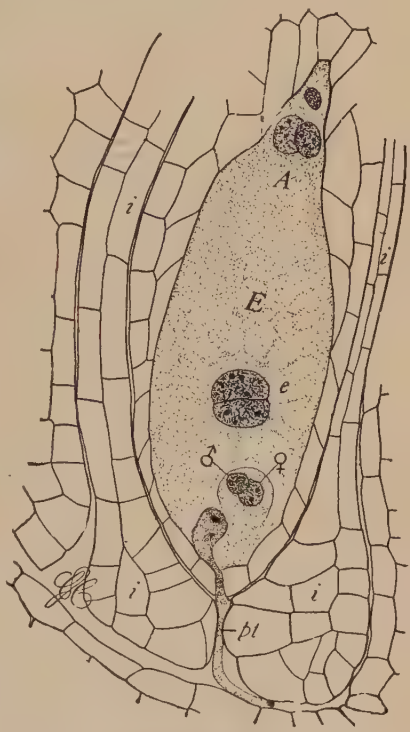

FIG. 80.—*Lilium philadelphicum.* × 335

Embryo-sac at time of fertilization. *A*, the three antipodals. *E*, protoplasm of the sac. *e*, polar nuclei fusing to form the endosperm nucleus; the male nucleus is about to fuse with the nucleus of the oösphere. *i*, the inner integument. *pt*, the pollen tube. Fixed in Carnoy's fluid and stained in cyanin and erythrosin. Fifteen microns thick. (From Bailey's Cyclopedia of American Horticulture.)

Later stages up to fertilization and the first divisions of the embryo may be stained as already described, but cyanin and erythrosin also give a particularly brilliant effect, especially after Carnoy's fluid. At the stage shown in Fig. 80 the male nucleus takes the cyanin, and the oösphere nucleus the erythrosin, although at a slightly later stage they stain alike. For the later stages in the development of the embryo, Delafield's hæmatoxylin is a far better stain.

Many of the Compositae, like *Aster*, *Taraxacum*, *Senecio*, and

Silphium, are excellent for a study of the mature sac and the formation of the embryo. The synergids in these forms are much more highly organized than in *Lilium*. The whole head may be cut if trimmed as indicated in Fig. 73, but for the embryo-sac at the fertilization period, and also for the development of the embryo, it is worth while to resort to the tedious process of dissecting the ovules out from the ovaries. Stages like those shown in Figs. 74–79 are small and rather unsatisfactory in Composites.

Many of the Ranunculaceae are easily studied. *Anemone patens*, var *Nuttalliana*, has a very beautiful embryo-sac, the egg, synergids, endosperm nucleus, and antipodals being rather large and sharply defined. *Hepatica*, *Caltha*, and some species of *Ranunculus* are exceptionally good.

Development of the Embryo.—The common shepherd's purse (*Capsella bursa-pastoris*) is a favorable form for a study of the development of a dicotyl embryo. The stages shown in Fig. 81, *A–F*, will be found in pods which are about one-eighth of an inch long. These may be put directly into the fixing agent, but stages like *G* and *H* are found in pods about three-sixteenths of an inch long, and such stages will be more readily fixed, infiltrated, and cut if the pods are trimmed, as shown in Fig. 73, *B*, before putting them into the fixing agent. Cut sections parallel to the flat face of the pod. Delafield's hæmatoxylin, without any contrast stain, gives the best results which we have secured.

For tracing the development of a monocotyl embryo *Sagittaria variabilis* can be recommended. Material is abundant, sections are easily cut, except in the latest stages, and it is not difficult to get a complete series. *Alisma plantago*, which is commonly figured in textbooks, is extremely hard to cut, especially in later stages. For these latest stages, it is better to take the dry, ripe seeds and germinate them. As soon as the embryo comes out, so that it can be separated from the hard seed coat, it is perfectly easy to fix and cut.

Leaves.—Where only a rapid examination is to be made, free-hand sections may be made in great numbers by using the method employed for pine needles. It is easy to get good sections of

leaves which can be got into paraffin, but it is difficult to get tender, succulent leaves into paraffin without distortion. Such leaves may be cut in celloidin.

For a study of the stomata, strip a piece of epidermis from the leaf, fix it, stain in Delafield's hæmatoxylin and erythrosin, pass

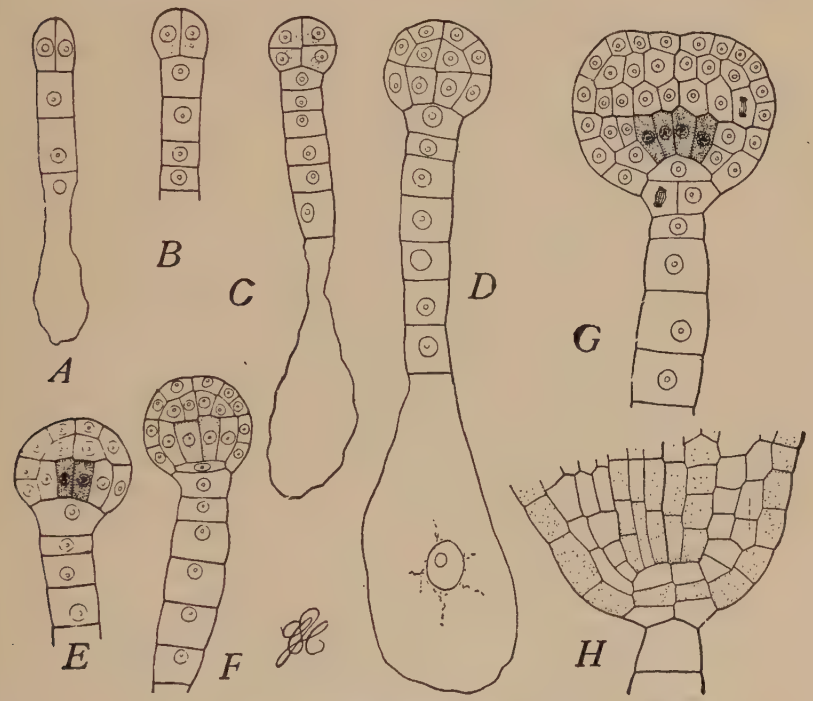

FIG. 81.—*Capsella bursa-pastoris.* × 400

A, first division in the embryo cell. *B*, quadrants. *C*, octants. *D*, dermatogen has been cut off. There are eight cells in the suspensor, the lower cell being very large and vesicular. *E*, differentiation in plerome and periblem. The plerome cells are shaded. *F*, the periblem of the root is completed at the expense of the upper cell of the suspensor. *G*, the mitotic figure in the suspensor cell indicates that the upper suspensor cell by a second contribution is about to complete the dermatogen of the root. *H*, plerome (shaded), periblem, dermatogen (shaded), and first layer of the root cap. Fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin. Ten microns thick.

it gradually through the alcohols, clear in xylol, and mount in balsam. Lily, tulip, hyacinth, and begonia may be suggested as favorable forms. Epidermis from the mature leaf of the common *Sedum pupurascens* will usually show stomata in all stages of development.

Stems and Roots.—The earlier stages in the development of vascular bundles in stems and roots are well shown in paraffin sections of young seedlings. The common bean is a favorable form, and it is easy to get material.

Most herbaceous stems and roots, and also the younger woody stems and roots, give the best results when cut in celloidin, as already described for *Pinus*. *Xanthium canadense* and *Ranunculus repens* can be recommended for a study of the vascular bundles. The cambium is very sharply brought out by Delafield's hæmatoxylin.

Petioles or leaf blades of *Nuphar* yield beautiful preparations. Fix for a few hours in a mixture of alcohol and formalin (100 c.c. of 70 per cent. alcohol and 2 c.c. of commercial formalin, a formula used by Dr. Lynds Jones). Stain in safranin and Delafield's hæmatoxylin. The sclerotic cells should take a brilliant red and the cellulose walls a rich purple.

Stems of petioles of the squash or pumpkin are to be preferred for demonstrating sieve tubes and companion cells. For the more minute details of the sieve plate it is best to cut out small pieces about one-fourth of an inch long and one-eighth of an inch square containing the vascular bundle. These pieces can be imbedded in paraffin.

For demonstrating the phellogen and the tissue developed from it, stems of *Geranium* or *Coleus* about one-fourth of an inch in diameter or seedlings of *Xanthium canadense* about three-sixteenths of an inch in diameter can be recommended. It is a good plan to take a piece from each of several successive internodes. If sections from each of these pieces be mounted in order under a single cover, the preparation will afford a valuable study of the origin and development of phellogen, the formation of intrafasicular cambium, and many other important features.

Sections may be cut free-hand or in celloidin.

The stem of Indian corn, imbedded in celloidin and stained in safranin and Delafield's hæmatoxylin, affords a good study of monocotyl stem anatomy.

Root-tips.— Besides showing the origin and development of the various root structures, the root-tip furnishes ever ready material for the study of mitosis. An onion thrown into a pan of water will soon send out numerous roots. Soak beans in water for several hours, and then plant them about an inch deep in loose, moist sawdust. The primary root will be long enough in two or three days. The large, flat beans are best. *Vicia faba* is very favorable. While such material is particularly available, the figures are not as satisfactory as those to be obtained in the root-tips of *Tradescantia virginica*, *Iris versicolor*, *Podyphyllum peltatum*, *Arisaema triphyllum*, *Cypripedium pubescens*, and many others. Transverse sections of young roots often show a remarkably regular arrangement of the cells, as can be seen in Fig. 82.

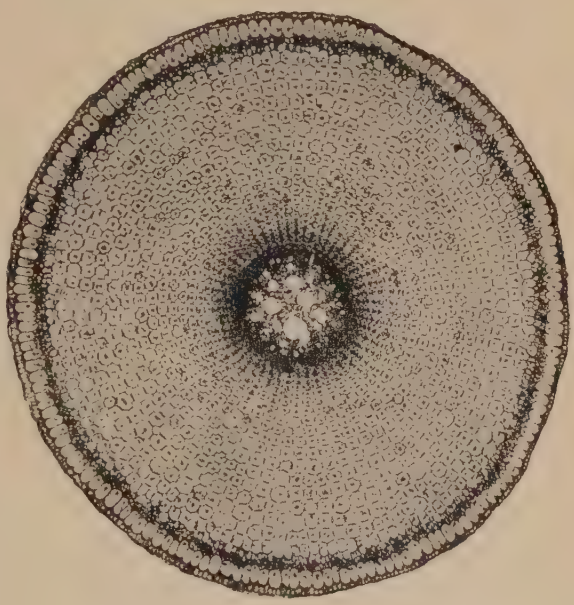

FIG. 82.—*Sparganium eurycarpum*. × 53
Transverse section of a root. Delafield's hæmatoxylin and acid fuchsin. Five microns.

Fix as directed for spermatogenesis and oögenesis. Miss Merriman[1] found that the chromatin is fixed better by mixtures containing osmic acid, while the linin shows more clearly in mixtures without osmic acid.

Doubtless cell division does not proceed with equal rapidity at all hours of the day. Kellicott[2] has shown that in the root-

[1] Merriman, Mabel L., Vegetative cell division in *Allium*. Botanical Gazette, **37**: 178–207. 1904.

[2] Kellicott, W. E., The daily periodicity of cell division and of elongation in the root of *Allium*. Bull. Torrey Bot. Club, **31**: 529–550. 1904.

tips of *Allium* there are in each twenty-four hours two periods at which cell division is at the maximum, and two at which it is at the minimum. The maximum periods are shortly before midnight (11 P. M.), and shortly after noon (1 P. M.). The minima, when cell division is at the lowest ebb, occur about 7 A. M. and 3 P. M. When cell division is most vigorous, there is little elongation, and when cell division is at the minimum, cell elongation is at the maximum. Consequently, root-tips of *Allium* should be collected about 1 o'clock P. M. or 11 o'clock P. M. It would be interesting to know whether these results are more general in their application.

We are painfully aware that the directions which have been given are very incomplete, but it is hoped that they will enable the student to devise for himself such methods as particular cases may demand.

CHAPTER XXIII

USING THE MICROSCOPE

The investigator who desires to see all that his microscope is capable of showing must study the optics of his instrument. The fundamental principles are presented in any good textbook of physics. Excellent practical hints are given in two booklets published by the leading American optical companies. These booklets tell the beginner how to set up the microscope, how to keep it in order, and give directions concerning illumination, dry and immersion objectives, mirror, condenser, diaphragm, and various other things. They were doubtless written for advertising purposes, but since they advertise by giving directions for securing the best results with the microscope, the information is very reliable. The Spencer Lens Co., of Buffalo, N. Y., and The Bausch and Lomb Optical Co., of Rochester, N. Y., furnish these booklets free of charge.

In the histological laboratory where preparations are being made, the microscope is in constant danger. A cheap microscope with a two-third objective and one ocular, such an instrument as can be got for $20 or less, can be used for examining preparations while they are wet with alcohols, oils, or other reagents. If it is necessary to use a better instrument for such work, cover the stage with a piece of glass—an old lantern slide is of about the right size—and be extremely careful not to get reagents upon the brass portions.

MICROMETRY

Everyone who desires to become at all proficient in the use of the microscope should learn to measure microscopic objects and should learn to form some estimate even without measuring, just as one guesses at the size of larger objects. In any measurement one should note the tube length, which is usually 160 mm. Where there is no revolving nosepiece, the draw-tube is simply pulled

out to the 160 mark. Where a nosepiece is used, its height should be measured, and the draw-tube should be pushed in from the 160 mark—a distance equal to the length of the nosepiece. There are in general use two practical methods of measuring

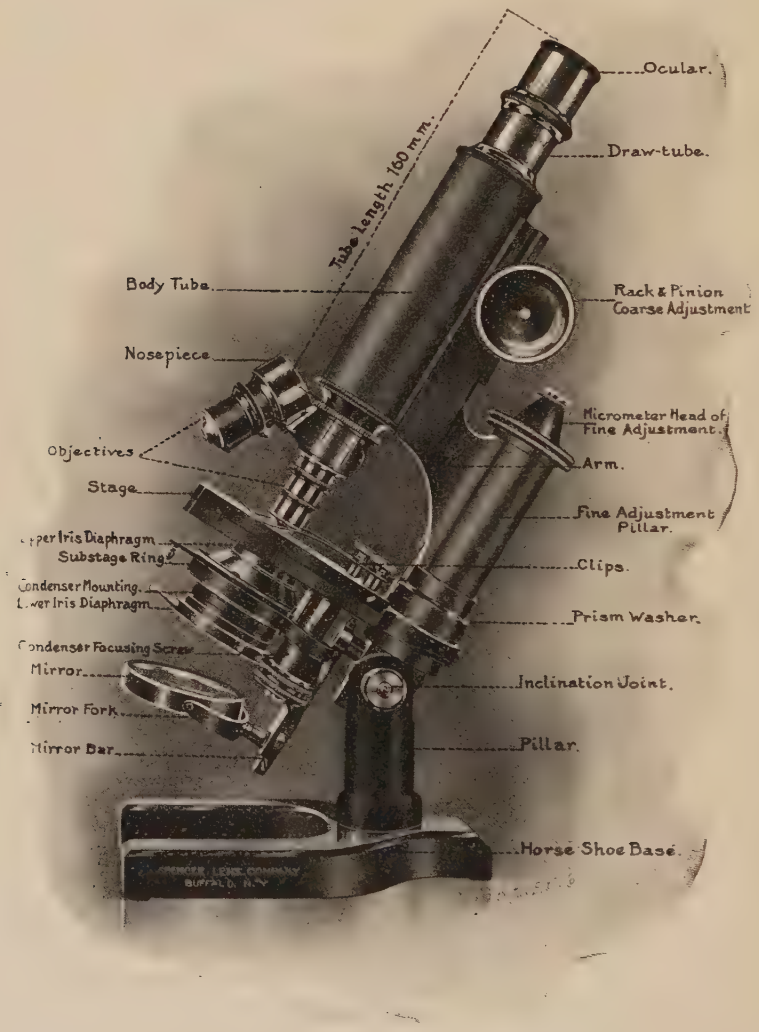

FIG. 83.—A modern microscope with the names of the various parts. From the Spencer Lens Co.'s booklet, "How to Use and Care for the Microscope."

microscopic objects, one by means of the ocular micrometer, and the other by means of camera lucida sketches.

Measuring with the Ocular Micrometer.—A stage micrometer and an ocular micrometer are necessary. A stage micrometer should be ruled in tenths and one hundredths of a millimeter. It does not matter what the spacing in the ocular micrometer may be, except that the lines must be at equal distances from each other. As a matter of fact, the ocular micrometer is generally ruled in tenths of a millimeter, but this ruling is more or less magnified by the lens of the ocular.

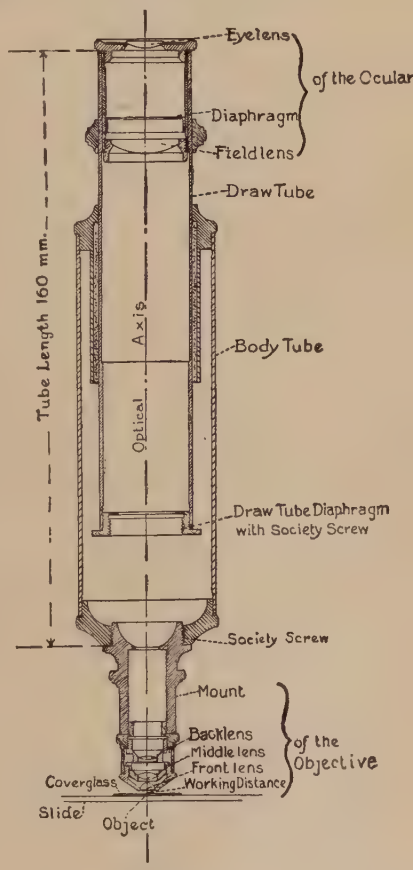

FIG. 84

Place the stage micrometer upon the stage and the ocular micrometer in the tube, and arrange the two sets of rulings so that the first line in the ocular micrometer will coincide with the first line of the stage micrometer, and then find the value of one space in the ocular micrometer. The method of finding this value is shown in the following case in which the tube length was 160 mm., the ocular a Zeiss ocular micrometer 2, and the objective a Leitz 3. Ninety-eight spaces in the ocular micrometer covered just fifteen of the larger spaces of the stage micrometer. Since the stage micrometer is ruled in tenths and one hundredths of a millimeter, the fifteen spaces equal 1.5 mm., or 1500μ.[1] Then 98 spaces of the ocular micrometer equal 1500μ; and one space in the ocular equals $\frac{1}{98}$ of

[1] One millimeter = 1000μ. The Greek letter, μ, is an abbreviation for $\mu\iota\kappa\rho\acute{o}\nu$, or micron.

1500 μ, or 15.3 μ. This value being determined, there is no further use for the stage micrometer. To measure the diameter of a pollen grain, put the preparation on the stage, using the same objective and ocular micrometer, and note how many spaces a pollen grain covers. If the pollen grain covers five spaces, its diameter is five times 15.3 μ, or 76.5 μ. In the same way, the value of a space in the ocular when used with the other objectives should be determined. The values for three or four objectives may be written upon an ordinary slide label and pasted upon the base of the microscope for convenient reference.

This method is the best one for measuring spores and for most measurements in taxonomy.

Measuring by Means of Camera Lucida Sketches.— This method is of great importance in research work, because various details can be measured with far greater rapidity than by the other method. Upon a piece of cardboard, about as thick as a postal card, draw a series of scales like those shown in Fig. 85.

Make a scale for each objective. It is not necessary to make scales for all the oculars, but only for the one in most constant use. It is absolutely necessary to note the tube length, length of the bar of the camera mirror, and inclination of the camera mirror, and the level at which the scale is made. A variation in any of these details will change the scale.

In using the stage micrometer, place the cardboard on the table, and with the aid of the camera lucida sketch the rulings of the micrometer. In Fig. 85 (which has been reduced by photography) note, for example, the scale drawn for Leitz objective 3. The spaces are drawn from the tenths of a millimeter rulings of the stage micrometer. Therefore each space on the card represents one-tenth of a millimeter, or 100 μ, and the ten spaces shown on the card represent 1 mm., or 1000 μ. By measuring with a metric rule the ten spaces upon the card, it is found that the scale is 114 mm. in length. The magnification of any drawing made with the same ocular and objective, under the same conditions, will therefore be 114 diameters. This does not mean that the magnifying power of Leitz objective 3 with Zeiss ocular 4 is

114 diameters, for the magnification of this combination is much less. A scale drawn at the level of the stage would show more nearly the magnifying power of the combination, but would still give too large a figure. The exact size of any object which has been sketched with this combination can now be measured by

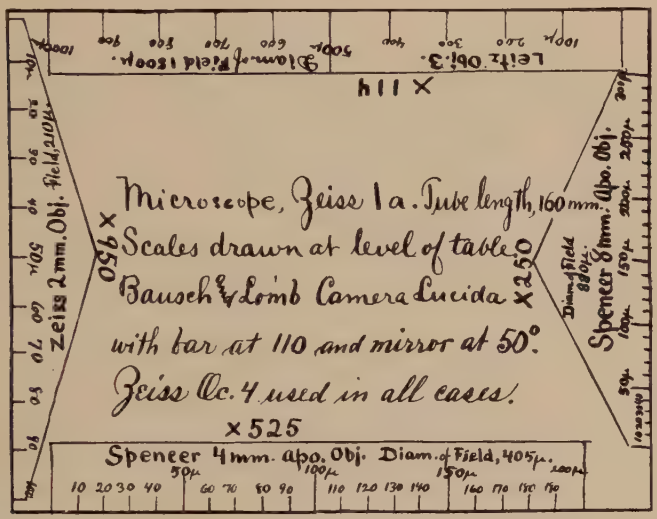

FIG. 85.—Scale card for practical use. All the data noted on the card are needed in practical work. The figure is considerably smaller than the scale card from which it was made.

applying the cardboard scale, just as one would measure gross objects with a rule.

The diameter of the field with this combination is 1800 μ. By knowing the diameter of the field with the various combinations, one can guess approximately the size of objects.

Other combinations are made in the same way. An excellent check on the accuracy of the computations is to measure the same object by means of the ocular micrometer and by the scale card. If the results are the same, the computations are correct.

In making sketches, it is a good plan to add the data which would be needed at any time in making measurement; e. g., L. 3, Z. oc. 4, table, 110, 50°, would show that the sketch was made

with Leitz objective 3, Zeiss ocular 4, at the level of the table, with mirror bar at 110, and camera mirror at 50°.

ARTIFICIAL LIGHT

During a considerable part of the year daylight is often insufficient for successful work with the microscope. Numerous

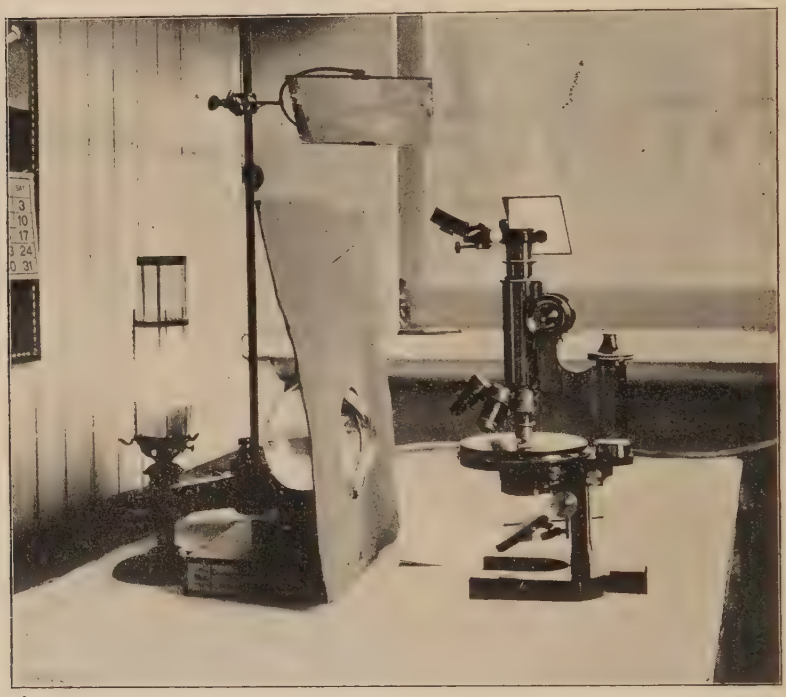

FIG. 86

contrivances for artificial illumination have been devised, some of them fairly good, but most of them thoroughly unsatisfactory. More than two hundred years ago, Hooke used a device for artificial illumination which probably suggested the apparatus now in use in Professor Strasburger's laboratory at Bonn. The apparatus in use in our own laboratory is only a slightly modified form of that used in the Bonn laboratory.

The apparatus consists, essentially, of a hollow sphere filled with liquid. A fairly good and practical light can be got with an ordinary lamp by allowing the light to pass through a wash bottle filled with a weak solution of ammonia copper sulphate. A piece of dark paper with a circular hole in it serves as a diaphragm, and at the same time protects the eyes from the direct light of the

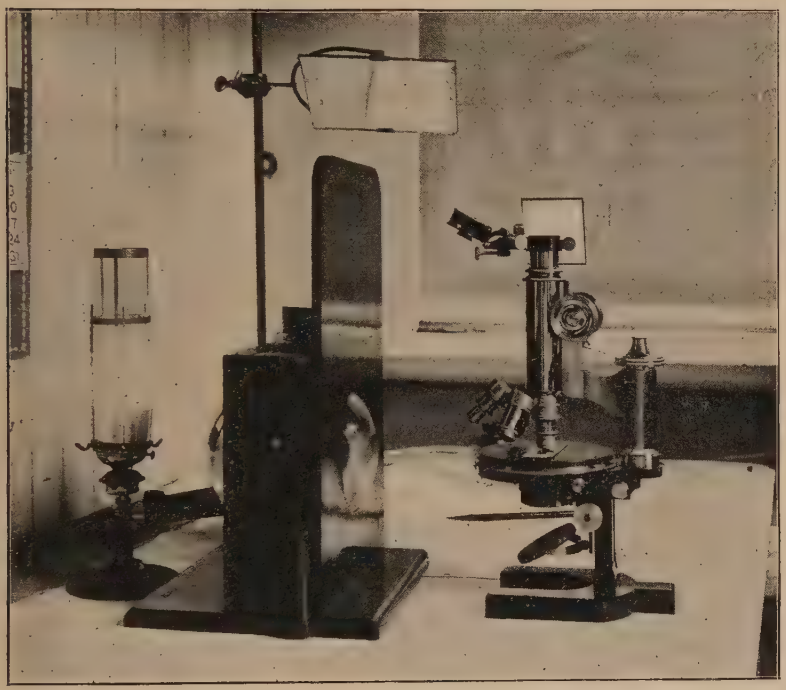

Fig. 87

lamp. Such an arrangement is shown in Fig. 86. Wash bottles, however, are not perfectly spherical and the mounting is not convenient. To secure a perfectly spherical globe, it was necessary to have a mold made. The globes, as we now use them, are of the finest flint glass, have a diameter of six inches, and are mounted in a convenient black frame, Fig. 87. The globe acts not only as a condenser, but also as a ray filter. For general laboratory work

and for nearly all research work, a weak solution of ammonia copper sulphate has proved most satisfactory. The solution (to fill one six-inch globe) may be made by adding 50 c.c. of ammonia to 25 c.c. of a 10 per cent. solution of copper sulphate, and then adding enough distilled water to fill the globe. If a white precipitate appears and makes the solution look milky, add more ammonia. The strength of the solution depends so much upon the power of the light that no fixed formula can be given. Simply dissolve in water a small crystal of copper sulphate—about as large as a grain of corn—then add about 50 c.c. of ammonia, and then add distilled water until a light, clear-blue solution is secured. With a very strong light, the solution may have a rather deep-blue color; with a less powerful light, the solution must be weaker.

In studying the extremely difficult achromatic structures concerned in nuclear division, a light violet solution of permanganate of potash is a good filter, if the preparation has been stained in violet. Similarly, various filters may be used according to the staining of the more critical structures.

The Welsbach lamp furnishes an excellent light. It should be placed so that the rays will be focused upon the mirror of the microscope. Some of the more powerful acetyline bicycle lamps are quite satisfactory. The Argand type of gas light is good, but will usually need a reflector behind it. A kerosene lamp must also be reinforced by a reflector. The old-fashioned silvered reflector, still used in country churches and halls, will do, but is hardly equal to the cheap reflectors of shorter focus which are so commonly used with incandescent electric lights. The incandescent electric light itself has not given satisfactory results. We have not tried the electric arc, but it would no doubt be satisfactory, if tempered by ground glass. It would probably be worth while to try the Nernst light.

When using the camera lucida, it is necessary to have a mirror placed so as to throw a fairly strong light upon the paper and the pencil point. A piece of silvered glass three or four inches square is large enough. Such a mirror can be held by an ordinary ring stand, as shown in Figs. 86 and 87.

These globes are being used in our laboratory both for research work and for ordinary class work. The globes and mounting were made according to our directions by Dr. William H. Knap, Powers Building, 156 Wabash Avenue, Chicago. For class work three or four globes are used at a table and receive their light from a single Welsbach lamp.

We believe that such light is not only equal to the best daylight, but is, in many cases, superior.

CHAPTER XXIV

LABELING AND CATALOGUING PREPARATIONS

THE LABEL

The labels shown in Fig. 71, on page 207, show as much as will generally be found desirable. The date of the collection of the material is often needed in addition. The date of making the preparation is of no value unless the student is testing the permanence of stains or something of that sort. It is hardly worth while to write upon the label the names of the stains used, for the student will soon learn to recognize the principal stains. We should say that the *first* thing to write upon a label is the genus and species of the plant; the next thing would be the name of the organ or tissue, and then might be added the date of collection; e. g., *Marchantia polymorpha*, young archegonia, April, 10, 1901. A hasty sketch on the label will often indicate any exceptionally interesting feature in the preparation. To facilitate finding such a feature, it is a good plan to mark the particular section or sections with ink, the marking being always on the underside of the slide so as not to cause any inconvenience if an immersion lens should be used.

CATALOGUING PREPARATIONS

As a collection grows, the student will need some device for locating readily any particular preparation. Some have their slides numbered and catalogued, but all devices of this sort are too cumbrous and slow for the practical worker in the laboratory. After several years' experience with a collection which now numbers about seven thousand preparations, the following method can be confidently recommended:

Four wooden slide boxes of the usual type will do for a beginning; they should be labeled: THALLOPHYTES, BRYOPHYTES, PTERIDOPHYTES, and SPERMATOPHYTES. As the collection grows

and new boxes are needed, the classification can be made more definite; e. g., there should be a box labeled BRYOYPHTES *Hepaticae* and one labeled BRYOPHYTES *Musci*. As the liverwort collection grows, three boxes will be necessary, and should be labeled BRYOPHYTES *Hepaticae* Marchantiales, BRYOPHYTES *Hepaticae* Jungermanniales, and BRYOPHYTES *Hepaticae* Anthocerotales. It will readily be seen that the process can be continued almost indefinitely, and that new slides may be at any time dropped into their proper places. A rather complete label gradually built up in this way is shown in Fig. 88:

BRYOPHYTES
HEPATICAE
Jungermanniales
Porella platyphyllum
Archegonia

FIG. 88

The beginner will often find that the mere placing of a slide in the proper box will refresh or increase his knowledge of classification. While this system is almost ideal for the careful worker, especially if he has some knowledge of classification, it is the worst possible method for a careless student, since a slide in the wrong box is almost hopelessly lost if the collection is large enough to need thorough classifying.

CHAPTER XXV

A CLASS LIST OF PREPARATIONS

Where a regular course in histology is conducted, it is a good plan to give each student at the outset a complete list of the preparations which he is expected to make. In a three-months' course a fairly representative collection of preparations can be made. The availability of material determines what a list shall be. Besides gaining an introduction to the use of the microscope and its accessories, a class meeting ten hours a week for twelve weeks should be able to do as much work as is outlined below.

In making the mounts the order indicated in the list should not be followed. Begin with temporary mounts, and then study, in succession, free-hand sections, the glycerine method, the Venetian turpentine method, the paraffin method, the celloidin method, and special methods. A large proportion of the time should be devoted to the paraffin method.

It is neither possible nor desirable that each student should in every case go through all the processes from collecting material to labeling. Some of the material may be in 70 per cent. alcohol, some in formalin, some in glycerine, and some in paraffin. One student may imbed in paraffin enough of the *Anemone* for the whole class, another may imbed the *Lilium* stamens, and by such a division of labor a great variety of preparations may be secured without a corresponding demand upon the time of the individual.

LIST OF PREPARATIONS

THALLOPHYTES

SCHIZOPHYTES

MYXOMYCETES

1. *Trichia varia.*—Paraffin sections, 5 μ. Safranin, gentian-violet, orange.

SCHIZOMYCETES

2. *Bacteria.*—*Coccus*, *Bacillus*, and *Spirillum* forms. Stain on cover-glass or slide.

3. *Bacillus anthracis.*—In liver of mouse. Paraffin sections, 5μ. Stain in gentian-violet, Gram's method.

CYANOPHYCEAE (SCHIZOMYCETES)

4. *Oscillaria.*—Put living material into 10 per cent. glycerine and allow it to concentrate.
5. *Tolypothrix.*—Use the Venetian turpentine method. Should show heterocysts, hormogonia, and false branching.
6. *Nostoc.*—Venetian turpentine method.
7. *Wasserblüthe.*—The principal forms in this material are:
   a) *Coelosphaerium Kützingianum.*—Colonies in the form of hollow spheres.
   b) *Anabaena gigantea.*—Filaments straight. Preparations should show vegetative cells, heterocysts, hormogonia, and spores.
   c) *Anabaena flos-aquae.*—Filaments curved. Stain on the slide and mount in balsam.
8. *Gloeotrichia.*—Smear on the slide, stain in safranin and gentian-violet, and mount in balsam; or use the Venetian turpentine method, staining in Magdala red and anilin blue and crushing under the cover-glass.

ALGÆ

CHLOROPHYCEAE

9. *Volvox.*—Use the Venetian turpentine method. If paraffin material is available, cut 5μ in thickness and stain in safranin, gentian-violet, orange.
10. *Scenedesmus.*—Let a drop containing the material dry upon the slide, stain in cyanin and erythrosin, and mount in balsam.
11. *Hydrodictyon.*—Use the Venetian turpentine method.

Each preparation should contain pieces of old and of young nets, and also at least one young net developing within an older segment. The greatest care must be taken not to injure the older segments while arranging the mount.

12. *Ulothrix.*—Use the Venetian turpentine method. Each mount should show various stages in the development of spores.
13. *Oedogonium.*—Stain in Magdala red and anilin blue and mount in Venetian turpentine.
14. *Coleochaete.*—Stain in Delafield's hæmatoxylin and mount in balsam.
15. *Cladophora.*—Stain some in iron-hæmatoxylin and some in Magdala red and anilin blue. Mount both together in Venetian turpentine.
16. *Diatoms.*—Make mounts of the frustules and also stained preparations showing the cell contents.

17. *Desmids.*—Make mounts of available forms. Use the Venetian turpentine method if material is sufficiently abundant.
18. *Zygnema.*—Stain in iron-hæmatoxylin and mount in Venetian turpentine.
19. *Spirogyra.*—Stain in Magdala red and anilin blue, and mount in Venetian turpentine.
20. *Vaucheria.*—Stain in Magdala red and anilin blue, and mount in Venetian turpentine.
21. *Chara.*—Cut paraffin sections of the apical cell, oögonia and antheridia.

PHAEOPHYCEAE

22. *Ectocarpus.*—Stain some in iron-hæmatoxylin and some in Magdala red and anilin blue. Mount both together in Venetian turpentine.
23. *Fucus vesiculosus.*—Antheridial conceptacle with paraphyses and antheridia; oögonial conceptacle with oögonia. Delafield's hæmatoxylin for paraffin sections.

RHODOPHYCEAE

24. *Batrachospermum* (or *Nemalion*).—Venetian turpentine method. Should show trichogyne, carpogonium, and cystocarp.
25. *Polysiphonia fibrillosa.*—Iron alum-hæmatoxylin or Magdala red and anilin blue. Mount in Venetian turpentine. Should show tetraspores, antheridia, and carpogonia.

FUNGI

PHYCOMYCETES

26. *Mucor stolonifer.*—Stain young sporangia in eosin, dilute Delafield's hæmatoxylin, or in Magdala red and anilin blue. Zygosporic material may be mounted without staining or after a *very* light staining in dilute Delafield's hæmatoxylin. Mount in Venetian turpentine.
27. *Saprolegnia.*—Stain in Magdala red and anilin blue. Mount in Venetian turpentine. Each mount should show sporangia and oögonia.
28. *Albugo* (*Cystopus*) *candidus* on *Cakile americana.*—Select white blisters which have not yet broken open. Paraffin, 5 μ. Safranin, gentian-violet, orange.
29. *Albugo bliti* on *Amarantus retroflexus.*—Cut out small portions of leaves in which the oögonia can be seen in abundance. Paraffin, 5 μ.

ASCOMYCETES

30. *Peziza.*—Paraffin sections of young apothecia, 5 μ or less; sections of older apothecia, 10 μ or 15 μ. Safranin, gentian-violet, orange.

31. *Aspergillus* (*Eurotium*).—Stain in eosin and mount in glycerine, or stain in Magdala red and anilin blue, and mount in Venetian turpentine.
32. *Penicillium.*—Treat like *Aspergillus.*
33. *Erysiphe commune* on *Polygonum aviculare.*—Strip the fungus from the leaf. Paraffin, 5 μ or less. Safranin, gentian-violet, orange.
34. *Uncinula necator* on *Ampelopsis quinquefolia.*—Stain in Magdala red and anilin blue. Mount whole in Venetian turpentine, and break the perithecia under the cover.
35. *Xylaria.*—Paraffin sections of younger stages. Delafield's hæmatoxylin and erythrosin. Be sure that some section in each mount shows the opening of a perithecium.

LICHENS

36. *Physcia stellaris.*—Cut in paraffin, 5 μ. Stain in cyanin and erythrosin.

BASIDIOMYCETES

37. *Puccinia graminis.*—Æcidium stage on barberry leaf. Paraffin. Cyanin and erythrosin. Uredospore and teleutospore stage in celloidin or paraffin.
38. *Coprinus comatus.*—Paraffin. Transverse sections of gills showing trama, paraphyses, basidia, and spores. To show the basidium with four spores, the sections should be 15 μ thick. For development of the spores, cut 5 μ or less. Safranin, gentian-violet, orange. *Boletus*, *Hydnum*, and *Polyporus* are treated in the same manner.

BRYOPHYTES

HEPATICAE

39. *Ricciocarpus natans.*—Paraffin, 10 μ or 15 μ. Delafield's hæmatoxylin. Archegonia, antheridia, and sporophytes imbedded in the gametophyte.
40. *Marchantia polymorpha.*—Paraffin, 5 or 10 μ. Archegonia, antheridia, and sporophytes.
41. *Anthoceros laevis.*—Paraffin, 5 or 10 μ. Longitudinal and transverse sections of sporophyte. Safranin, gentian-violet, orange.
42. *Pellia epiphylla.*—Paraffin, 5 or 10 μ. Longitudinal sections of sporophyte attached to gametophyte. Safranin, gentian-violet, orange.
43. *Porella platyphyllum.*—Paraffin, 10 μ. Delafield's hæmatoxylin. Archegonia, antheridia, sporophyte, and apical cell.

MUSCI

44. *Sphagnum.*—Leaf buds. Safranin and Delafield's hæmatoxylin. Tease and mount in balsam.

45. *Sphagnum.*—Capsule. Paraffin. Delafield's hæmatoxylin and erythrosin.
46. *Funaria hygrometrica.*—Paraffin. Longitudinal and transverse sections of young capsules. Delafield's hæmatoxylin.
47. *Funaria hygrometrica* or any favorable form. Protonema. Place the well-cleaned material directly into 10 per cent. glycerine and allow it to concentrate. Mount in glycerine or glycerine jelly.
48. *Bryum proliferum.*—Paraffin. Antheridia, 10 μ; archegonia, 15 to 20 μ; capsule, 10 μ.

PTERIDOPHYTES

FILICALES

49. *Aspidium.*—Cut in paraffin, 10 μ. Stain in safranin, gentian-violet, orange.
50. *Asplenium Thelypteris.*—Stain in eosin or erythrosin and mount pinnules whole in balsam.
51. *Adiantum.*—Mount prothallia whole in Venetian turpentine. Each mount should contain both male and female prothallia. Make paraffin sections of antheridia, archegonia and young embryos.
52. *Pteris cretica.*—Transverse sections of leaves with sori.
53. *Pteris aquilina.*—Transverse and longitudinal sections of rhizome. Stain in safranin and Delafield's hæmatoxylin, and also in methyl green and acid fuchsin.
54. *Lygodium.*—Mount entire sporangia without staining. Balsam or Venetian turpentine.
55. *Osmunda.*—Cut sections of sporangia, 5 μ. Stain for mitotic figures.
56. *Marsilea.*—Paraffin. Longitudinal and transverse sections of young sporocarps. Sections of mature microspores, megaspores, and of young embryos.
57. *Azolla carolinense.*—Vertical sections of the entire plant showing microsporangia and megasporangia. Safranin, gentian-violet, orange.
58. *Botrychium virginianum.*—Paraffin. Stain rhizome, stipes, and root in safranin and Delafield's hæmatoxylin. Stain sporangia in iron-hæmatoxylin.

EQUISETALES

59. *Equisetum arvense.*—Prothallia in Venetian turpentine. Stem tips in paraffin. Transverse section of stem in celloidin.

LYCOPODIALES

60. *Lycopodium lucidulum.*—Transverse section of stem. Free-hand or in celloidin. Safranin and Delafield's hæmatoxylin.
61. *Lycopodium inundatum.*—Paraffin. Longitudinal sections of strobilus.

62. *Selaginella.*—Paraffin. Longitudinal sections of rather mature strobili. Cyanin and erythrosin, or safranin, gentian-violet, orange.
63. *Isoetes echinospora.*—Transverse section of stem. Paraffin. Safranin and Delafield's hæmatoxylin.
64. *Isoetes echinospora.*—Paraffin. Longitudinal sections of microsporangia and megasporangia. Safranin, gentian-violet, orange.

SPERMATOPHYTES

GYMNOSPERMS

CYCADALES

65. *Zamia.*—Longitudinal section of the ovule, 10 μ. Safranin, gentian-violet, orange. Transverse sections of microsporophyll.

GINKGOALES

66. *Ginkgo biloba.*—Longitudinal sections of endosperm showing archegonia or young embryos. Paraffin 10 μ.
Sections of microsporangia with nearly mature pollen, 5 μ.

CONIFERALES

67. *Pinus Laricio.*—Transverse section of needles and young stem. Free-hand or celloidin. Safranin and Delafield's hæmatoxylin.
68. *Pinus strobus.*—Free-hand sections of well-seasoned wood. Methyl green and fuchsin, or safranin and Delafield's hæmatoxylin.
69. *Pinus Laricio.*—Paraffin. Ovule with archegonia. Safranin, gentian-violet, orange.
70. *Pinus sylvestris* or *P. Laricio.* Paraffin. Embryos. Cyanin and erythrosin.
71. *Pinus Laricio.* Paraffin. Longitudinal section of mature staminate strobilus. Safranin, gentian-violet, orange.

GNETALES

72. Longitudinal section of the ovule of *Ephedra.*

ANGIOSPERMS

MONOCOTYLS

73. *Alisma.*—Longitudinal sections of embryo from germinating seed.
74. *Zea Mais.*—Celloidin. Longitudinal and transverse sections of stem.
75. *Tradescantia virginica.*—Paraffin, 5 to 10 μ. Longitudinal sections of root. Stain for mitosis.
76. *Allium*—Paraffin. Transverse sections of older roots for vascular system.
77. *Lilium philadelphicum.*—Paraffin, 10 μ. Transverse sections of ovary; transverse sections of anthers.

78. *Lilium longiflorum.*—Strip epidermis from leaf and stain for stomata. Cut sections of leaf in paraffin, 10 to 20 μ.
79. *Smilax herbacea.*—Celloidin. Transverse section of root. Safranin and Delafield's hæmatoxylin.
80. *Iris.*—Longitudinal sections of anther. Paraffin, 5 μ. Stain for mitotic figures.

DICOTYLS

81. *Nuphar advena.*—Celloidin. Section of leaf 15 to 40 μ thick. Safranin and Delafield's hæmatoxylin.
82. *Anemone patens.*—Paraffin. Embryo-sac.
83. *Capsella bursa-pastoris.*—Paraffin. Floral development, 5 μ. Embryos, 5 to 10 μ. Stain both in Delafield's hæmatoxylin without any contrast stain.
84. *Pelargonium.*—Transverse sections of stem to show phellogen and intrafasicular cambium. Free-hand or celloidin.
85. *Tilia americana.*—Celloidin or free-hand. Transverse sections of small stems one-eighth to one-fourth of an inch in diameter. Safranin and Delafield's hæmatoxylin.
86. *Silphium.*—Longitudinal sections of the ovule at the fertilization period.
87. *Taraxacum officinale.*—Paraffin. Floral development, 5 μ. Embryo-sac, 10 to 15 μ.

CHAPTER XXVI

FORMULÆ FOR REAGENTS

FIXING AGENTS

Absolute Alcohol.—Used alone without any mixtures.

Carnoy's Fluid.—

Absolute alcohol	6 parts
Chloroform	3 parts
Glacial acetic acid	1 part

Farmer's Fluid.—

Absolute alcohol	2 parts
Glacial acetic acid	1 part

Formalin Alcohol (Lynds Jones's formula).—

70 per cent. alcohol	100 c.c.
Commercial formalin	2 c.c.

Stock Chromo-Acetic Solution.—

Chromic acid	1 g.
Glacial acetic acid	1 c.c.
Water	100 c.c.

Schaffner's Chromo-Acetic Solution.—

Chromic acid	0.3 g.
Glacial acetic acid	0.7 c.c.
Water	99 c.c.

Chromo-Acetic Solution (for delicate structures).—

Chromic acid	1 g.
Glacial acetic acid	3 c.c.
Water	300 c.c.

The addition of 10 drops of osmic acid to 50 c.c. of this solution is often an advantage.

Chromo-Acetic Solution (for marine algæ).—

Chromic acid	1 g.
Glacial acetic acid	0.4 c.c.
Sea-water	400 c.c.

Material must be washed in sea-water.

Flemming's Fluid (weaker solution).—

A	1 per cent. chromic acid	25 c.c.
	1 per cent. acetic acid	10 c.c.
	Water	55 c.c.
B	1 per cent. osmic acid	55 c.c.

Keep the mixture A made up, and add B as the reagent is needed for use, since it does not keep well.

Flemming's Fluid (stronger solution).—

1 per cent. chromic acid	45 c.c.
2 per cent. osmic acid	12 c.c.
Glacial acetic acid	3 c.c.

Merkel's Fluid.—

1.4 per cent. solution of chromic acid	25 c.c.
1.4 per cent. solution of platinic chloride	25 c.c.

Hermann's Fluid.—

1 per cent. platinic chloride	15 parts
Glacial acetic acid	1 part
2 per cent. osmic acid	4 or 2 parts

Picric Acid.—

Picric acid	1 g.
Water or 70 per cent. alcohol	100 c.c.

Corrosive Sublimate and Acetic Acid.—

Corrosive sublimate	3 g.
Glacial acetic acid	3 c.c.
70 per cent. alcohol (or water)	100 c.c.

Corrosive Sublimate, Acetic Acid, and Picric Acid.—

Corrosive sublimate	5 g.
Glacial acetic acid	5 c.c.
Picric acid	1 g.
50 per cent. alcohol	100 c.c.

Corrosive Sublimate and Picric Acid (Jeffrey's formula).—

Corrosive sublimate, saturated solution in 30 per cent. alcohol	3 parts
Picric acid, saturated solution in 30 per cent. alcohol	1 part

Formalin (weaker solution).—

Formalin	2 c.c.
Water	98 c.c.

Formalin (stronger solution).—

Formalin	4 c.c.
Water	100 c.c.

Osmic Acid.—

Osmic acid	1 c.c.
Distilled water	100 c.c.

The bottle in which the solution is to be kept, and also the glass tube in which the acid is sold, must be thoroughly cleaned. Break off the end of the tube, and drop both tube and acid into the distilled water, or simply drop the tube into the bottle and shake the bottle until the tube breaks.

STAINS

Delafield's Hæmatoxylin.—"To 100 c.c. of a saturated solution of ammonia alum add, drop by drop, a solution of 1 g. of hæmatoxylin dissolved in 6 c.c. of absolute alcohol. Expose to air and light for one week. Filter. Add 25 c.c. of glycerine and 25 c.c. of methyl alcohol. Allow to stand until the color is sufficiently dark. Filter and keep in a tightly stoppered bottle." (Stirling and Lee.)

The solution should stand for at last two months before it is ready for using.

Erlich's Hæmatoxylin.—

Distilled water	50 c.c.
Absolute alcohol	50 c.c.
Glycerine	50 c.c.
Glacial acetic acid	5 c.c.
Hæmatoxylin	1 g.
Alum in excess.	

Keep it in a dark place until the color becomes a deep red. If well stoppered, it will keep indefinitely.

Boehmer's Hæmatoxylin.—

A	Hæmatoxylin	1 g.
	Absolute alcohol	12 c.c.
B	Alum	1 g.
	Distilled water	240 c.c.

The solution A must ripen for two months. When wanted for use, add about 10 drops of A to 10 c.c. of B. Stain 10 to 20 minutes. Wash in water and proceed as usual.

Mayer's Hæm-Alum.—Hæmatoxylin, 1 g., dissolved with heat in 50 c.c. of 95 per cent. alcohol and added to a solution of 50 g. of alum in a liter of distilled water. Allow the mixture to cool and settle; filter; add a crystal of thymol to preserve from mold. (Lee.)

It is ready for use as soon as made up. Unless attacked by mold, it keeps indefinitely.

Haidenhain's Iron-Hæmatoxylin.—This stain was introduced by Haidenhain in 1892 and has gained a well-deserved popularity with those engaged in cytological work. Two solutions are used, and they are never mixed:

A. One and one-half to 4 per cent. aqueous solution of ammonia sulphate of iron. (At present we use a 3 per cent. solution.)

B. One-half per cent. aqueous solution of hæmatoxylin.

Greenacher's Borax Carmine.—

Carmine	3 g.
Borax	4 g.
Distilled water	100 c.c.

Dissolve the borax in water and add the carmine, which is quickly dissolved with the aid of gentle heat. Add 100 c.c. of 70 per cent. alcohol and filter. (Stirling.)

Alum Carmine.—A 4 per cent. aqueous solution of ammonia alum is boiled 20 minutes with 1 per cent. of powdered carmine. Filter after it cools. (Lee.)

Alum Cochineal.—

Powdered cochineal	50 g.
Alum	5 g.
Distilled water	500 c.c.

Dissolve the alum in water, add the cochineal, and boil; evaporate down to two-thirds of the original volume and filter. Add a few drops of carbolic acid to prevent mold. (Stirling.)

Picro-Carmine.—

Picro-carmine (picro-carminate of ammonia)	1 g.
Water	100 c.c.

Eosin.—

Eosin	1 g.
Water, or 70 per cent. alcohol	100 c.c.

General Formula for Anilins.—Make a 3 per cent. solution of anilin oil in distilled water; shake well and frequently for a day; add enough alcohol to make the whole mixture about 20 per cent. alcohol; add 1 g. of cyanin, erythrosin, safranin, gentian-violet, etc., to each 100 c.c. of this solution.

Cyanin.—This general formula is not at all successful with Grüber's cyanin, but gives satisfactory results with an immensely cheaper cyanin, sold by H. A. Metz & Co., 122 Hudson Street, New York.

Anilin Blue.—

Anilin blue	1 g.
85, or 90 per cent. alcohol	100 c.c.

For staining before mounting in Venetian turpentine, this stain should be made up in strong alcohol, even if the dry stain is intended for aqueous solution.

Iodine Green.—

Iodine green	1 g.
70 per cent. alcohol	100 c.c.

Methyl Green.—

Methyl green	1 g.
Glacial acetic acid	1 c.c.
Water	100 c.c.

If the preparation is to be mounted in balsam, a slight trace of acetic acid and also a trace of methyl green should be added to the absolute alcohol used for dehydrating.

For staining vascular bundles, the acid may be omitted, even from the formula.

Fuchsin.—

Fuchsin	1 g.
95 per cent. alcohol	100 c.c.
Water	100 c.c.

Acid Fuchsin.—

Acid fuchsin	1 g.
Water	100 c.c.

Use this formula when staining woody tissues in methyl green and acid fuchsin.

Ziehl's Carbol Fuchsin.—

Fuchsin	1 g.
Carbolic-acid crystals	5 g.
95 per cent. alcohol	10 c.c.
Water	100 c.c.

Fuchsin and Iodine Green Mixtures.—Two solutions are kept separate, since they do not retain their efficiency long after they are mixed.

A	Fuchsin (acid)	0.1 g.
	Distilled water	50 c.c.
B	Iodine green	0.1 g.
	Distilled water	50 c.c.
C	Absolute alcohol	100 c.c.
	Glacial acetic acid	1 c.c.
	Iodine	0.1 g.

Stain in equal parts of A and B. Transfer from the stain directly to solution C, and from C to xylol.

Another Formula.—

A	Acid fuchsin	0.5 g.
	Water	100 c.c.
B	Iodine green	0.5 g.
	Water	100 c.c.

Mix a pipettefull of A with a pipettefull of B; stain 2–8 minutes; dehydrate rapidly and mount in balsam.

Magdala Red.—

Magdala red	1 g.
85 or 90 per cent. alcohol	100 c.c.

Use this formula when staining in Magdala red and anilin blue, before mounting in Venetian turpentine.

Safranin.—

Safranin	1 g.
95 per cent. alcohol	50 c.c.
Water	50 c.c.

Gentian-Violet.—

Gentian-violet	1 g.
95 per cent. alcohol	20 c.c.
Water	80 c.c.
Anilin oil	3 c.c.

A 1 per cent. solution in water keeps better.

Pyoktanin.—This is sold by E. Merck, in Darmstadt, Germany. Dissolve 1 g. of pyoktanin in 30 c.c. of water.

Orange G.—

Orange G	1 g.
Water	100 c.c.

Bismarck Brown.—

Bismarck brown	2 g.
70 per cent. alcohol	100 c.c.

Nigrosin.—

Nigrosin	1 g.
Water	100 c.c.

Gram's Solution.—

Iodine	1 g.
Iodide of potassium	2 g.
Water	300 c.c.

MISCELLANEOUS

Schultze's Maceration Fluid.—The ingredients are nitric acid and potassium chlorate. They are mixed only as the reagent is applied. See chapter on "Special Methods."

Iodine (solution for starch test).—Dissolve 1 g. potassium iodide in 100 c.c. of water and add 0.3 g. sublimed iodine.

Fehling's Solution.—Keep it in three bottles labeled A, B, and C.

A	Cupric sulphate	3 g.
	Water	100 c.c.
B	Sodium potassium tartrate (Rochelle salts)	16 g.
	Water	100 c.c.
C	Caustic soda	12 g.
	Water	100 c.c.

When needed for use, add 10 c.c. of water to 5 c.c. from each of the three bottles.

Millon's Reagent.—

Mercury	1 c.c.
Concentrated nitric acid	9 c.c.
Water	10 c.c.

Cuprammonia.—Prepare by pouring 15 per cent. ammonia water upon copper turnings or filings. Let it stand in an open bottle.

Phloroglucin.—Use a 5 per cent. solution in water or alcohol.

Celloidin.—To make a 2 per cent. solution, add one tablet of Schering's celloidin and enough ether-alcohol (equal parts absolute alcohol and ether) to make the whole weigh 2000 g.

Where only a small quantity is needed, shave off 2 g. of celloidin and add 100 c.c. of ether alcohol.

Eycleshymer's Clearing Fluid.—Mix equal parts of bergamot oil, cedar oil, and carbolic acid.

Venetian Turpentine.—To make a 10 per cent. solution, add 90 c.c. of absolute alcohol to 10 c.c. of thick Venetian turpentine. Stir it with a glass rod. Guess at the amount of turpentine, for it is not easy to clean things which have contained Venetian turpentine.

The Following need no formulæ: Acetic acid, hydrochloric acid, nitric acid, sulphuric acid, carbolic acid, chloroform, ether, xylol, cedar oil, clove oil, bergamot oil, turpentine, glycerine, paraffin, balsam.

AMOUNTS OF REAGENTS REQUIRED

It is difficult to estimate the amounts of the various reagents needed by a class in histology. Two dangers must be guarded against—wastefulness and too great economy; for economy in some reagents, like absolute alcohol, turpentine, xylol, and clove oil, may be so rigid as to make the preparations decidedly inferior.

Each Student should have some reagents upon his own table. The following is an estimate of the amounts of some reagents used by each student in a three-months' course in method: commercial alcohol (about 95 per cent.), 3 liters; absolute alcohol, 400 c.c.; turpentine (for dissolving paraffin ribbons), 200 c.c.; xylol, 200 c.c.; clove oil, 75 c.c.; Canada balsam, 25 c.c.; hard and

soft paraffin, 400 g. each; safranin, gentian-violet, orange, cyanin, erythrosin, Delafield's hæmatoxylin, iron-hæmatoxylin, and ammonia sulphate of iron (3 per cent.), 100 c.c. each.

For General Use of the entire class, other stains and reagents may be kept upon a table accessible to all. Some stains which act very rapidly, like cyanin, erythrosin, and orange, may be kept upon the common table. A class of ten will use about 20 liters of the stock solution of chromo-acetic acid, and of glacial acetic acid about 400 c.c.; commercial formalin, about 200 c.c.; Venetian turpentine, 500 c.c.; cedar oil, 200 c.c.; Eycleshymer's clearing fluid, 100 c.c.; glycerine, 600 c.c.; chloroform, 100 c.c.; ether-alcohol, 200 c.c.; celloidin, 40 g.; hydrochloric acid, 200 c.c.; nitric acid and sulphuric acid, 50 c.c. each; Magdala red and methyl green, 200 c.c. each; other stains, 100 c.c. each.

No attempt has been made to make the above list absolutely complete. The equipment of any laboratory will be built up gradually. When the microtome needs oiling, some sewing-machine oil can be added to the general equipment. It is hoped that reagents omitted from the list are, like the sewing-machine oil, readily secured without vexatious delays.

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